

STEROL REGULATION OF HEPATIC LIPASE.
A NOVEL ROLE FOR STEROL REGULATORY ELEMENT BINDING PROTEIN
IN CHOLESTEROL HOMEOSTASIS

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by
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Cornell University 2006

Hepatic lipase (HL) plays a critical role in the modulation of plasma lipoprotein concentrations and specifically the concentrations of high density lipoproteins and small low density lipoproteins. HL is likely to play a role in cellular cholesterol homeostasis. Despite numerous studies aimed at characterizing HL regulation, the role of cholesterol in controlling HL expression and/or activity levels remains unclear. In Fu5AH and McA-RH7777 rat hepatoma cells, cholesterol treatment resulted in a marked decrease in secreted rat HL (rHL) mass. Similarly, the acyl-CoA:cholesterol acyltransferase inhibitor 58-035 decreased rHL secretion, suggesting that unesterified cholesterol mediated the downregulation. Cholesterol alone or in combination with 25-hydroxy-cholesterol, or 58-035 decreased rHL mRNA levels without affecting mRNA degradation rate. Sterol Regulatory Element Binding Proteins (SREBPs) are the major transcription factors mediating the feedback regulation of cholesterol levels. Sterol treatments decreased nuclear SREBPs in human HepG2 cells without affecting the activity of a -1480/+14 human HL (hHL) promoter luciferase construct in rat or human cells. In HepG2 cells, statin (compactin) treatment or over-expression of nuclear SREBP1a (nSREBP1a) decreased the activity of a -117/+14 hHL promoter construct and HL mRNA levels. Forced expression of nSREBP1a reversed the Upstream Stimulatory Factor 1 (USF1)-mediated activation of hHL promoter constructs. Gel-shift and supershift assays identified binding sites for Hepatocyte Nuclear Factor 1 (HNF1), HNF4 and USF1 within the hHL promoter at -70/-48, -252/-218, and -317/-298 respectively. Binding of these factors was diminished using nuclear extracts from sterol or compactin treated cells. No direct binding of nSREBP

to the hHL promoter was identified. SREBP1a bound to USF1 in co-immunoprecipitation experiments, suggesting inhibition of HL transcription by cholesterol or compactin may occur through SREBP1 interaction with USF1 or its co-activators. In rat cells, cholesterol or 58-035 decreased rHL protein synthesis while protein turnover was unchanged. In vitro translation assays demonstrated a decrease in HL translation efficiency in sterol-treated cytoplasmic extracts. These experiments provide evidence for a novel aspect of the function of SREBPs in the crosstalk between cholesterol and fatty acid/triglyceride metabolism.

BIOGRAPHICAL SKETCH

Anne Delphine Le Bourg was born in Tours, France, and soon moved back to her roots in Brittany where she grew up in Rennes. Her amazingly supportive parents and traumatizing brother helped her enjoy a very full and fun childhood. During high school at Émile Zola she specialized in Sciences but spent the major part of her time focusing on about everything else, with most of her studying time devoted to learning Mandarin. Being interested in a career in biology, she decided for reasons still unclear to her at this time to steer clear of the more common, less stressful French University path and entered a Classe Préparatoire aux Grandes Écoles at Châteaubriand in her hometown. After two years of rather intense studying in BioSup-BioSpé, she was accepted in the École Nationale Supérieure d'Agronomie de Rennes where she spent the next three years studying agronomical engineering. There, she managed to keep Chinese as her third language through an agreement with a business school. Keeping research in mind, she registered for a dual program in her third year to take her Diplôme d'Études Approfondies (5th year University degree) along with completing her Master's in Engineering. That same year, two research internships in Dr. Bensadoun's lab at Cornell and in Dr. Pfeffer's group at Stanford were enriching enough that she decided to decline her merit-based fellowship for a PhD in France and cross the Atlantic. She chose to join Dr. André Bensadoun's group and study lipid metabolism. There, among other great experiences, she met her amazing husband, Zhen Huang. Somewhat ironically, after seven years of learning mandarin, she realized she still could not speak two words of Chinese with her spouse whose home language is Taishanese, a dialect of Cantonese. For the past few years, she has concentrated on hepatic lipase.

To Zhen -

*In memory of the innumerable horrendous bus rides to visit me,
including all the 24-hour round trips in between Ithaca and Boston,
they were ALL worth it=)*

To my parents, Elisabeth and Michel -

*Thanks for everything, obviously, and more recently
thank you for all of your visits and our weekly phone conferences
You're both great!!! - Don't ever change!*

*“... Passe, il faut que tu poursuives,
Cette belle ombre que tu veux”
Clotilde, Alcools, Guillaume Apollinaire*

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LIST OF COMMONLY USED ABBREVIATIONS

ABCA1	ATP-binding cassette transporter A1
ACAT	acyl CoA : cholesteryl acyltransferase
ALLN	N-acetyl-leucyl-leucyl-norleucinal
Apo	apolipoprotein
ATP	adenosine 5'-triphosphate
bHLH	basic helix-loop-helix
bp	base pair(s)
BSA	bovine serum albumin
CAD	coronary artery disease
cAMP	adenosine 3',5'-cyclic monophosphate
CAT	chloramphenicol acetyltransferase
CDCA	chenodeoxycholic acid
cDNA	complementary DNA
CE	cholesterol ester(s)
CETP	cholesteryl ester transfer protein
CHAPS	3-(3-cholamidopropyl)diethyl-ammonio-1 propanesulfonate
ChIP	chromatin immunoprecipitation
CHX	cycloheximide
cpm	counts per minute
CVD	cardiovascular disease
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FA	fatty acid(s)
FBS	fetal bovine serum
FC	free cholesterol
FCS	fetal calf serum
FFA	free fatty acid(s)
FXR	farnesoid X receptor
GFP	green fluorescent protein
HDL	high density lipoprotein
HDL-C	high density lipoprotein - cholesterol
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
hHL	human hepatic lipase
HL	hepatic lipase
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HNF	hepatocyte nuclear factor
HRP	horseradish peroxidase
HSPG	heparan sulfate proteoglycan

IDL	intermediate density lipoprotein
IgG	immunoglobulin G
IVT	in vitro translation
kb	kilobase(s)
kDa	kilodalton
LCAT	lecithin : cholesterol acyltransferase
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
LPDS	lipoprotein-deficient serum
LPL	lipoprotein lipase
LRP	low density lipoprotein receptor- related protein
LXR	liver X receptor
m β -CD	methyl-beta-cyclodextrin
MEM	Eagle's minimal essential medium
mRNA	messenger RNA
NIDDM	non-insulin dependent Diabetes Mellitus, type II diabetes
nSREBP	nuclear SREBP, mature transcription factor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PL	phospholipid
PMSF	phenylmethylsulfonyl fluoride
PPAR	peroxisome proliferator-activated receptor
pSREBP	precursor SREBP
PUFA	polyunsaturated fatty acid(s)
RCT	reverse cholesterol transport
rHL	rat hepatic lipase
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse-transcription polymerase chain reaction
RXR	retinoid X receptor
SCAP	SREBP-cleavage-activating protein
SDS	sodium dodecyl sulfate
siRNAs	small interfering RNAs
SRE	sterol responsive element
SREBP	sterol regulatory element binding protein
TC	total cholesterol
TCA	trichloroacetic acid
TG	triglyceride(s) / triacylglycerol
USF	upstream stimulatory factor
VLDL	very low density lipoprotein

Chapter 1 - Review of literature

High cholesterol levels are associated with increased risk for cardiovascular disease. Hepatic lipase (HL), an enzyme which is active in the liver and to a lesser extent in the adrenals, ovaries and in macrophages, is implicated in cholesterol metabolism at multiple levels. Its roles in lipoprotein metabolism stem from both its enzymatic role as a phospholipase and a triglyceride hydrolase and from its “bridging” role to increase the residency time of lipoproteins in close proximity to their cellular receptors. Its combined role as a lipolytic enzyme and as a ligand increases lipoprotein uptake and cholesterol loading in cell culture. By increasing cholesterol uptake, HL affects cell cholesterol levels, and it could be part of a feedback mechanism to maintain stable cholesterol levels within the cell. Because HL may play a role in maintaining cellular cholesterol homeostasis, the issue of HL regulation by cholesterol is of particular interest.

Within liver cells, intracellular cholesterol homeostasis is tightly maintained. Sterol Regulatory Element Binding Proteins (SREBPs) are the major transcription factors mediating the feedback regulation of cholesterol levels by controlling the expression of genes involved in cholesterol biosynthesis, lipoprotein uptake, or lipoprotein hydrolysis. Therefore, it became apparent that SREBPs may also play a significant role in the cholesterol regulation of HL expression.

The goal of this thesis is to investigate the regulation of HL by cholesterol and factors which affect intracellular cholesterol levels such as SREBPs.

1.1 *Hepatic lipase*

1.1.1 Characteristics

Hepatic lipase (HL) belongs to a family of lipases that also includes pancreatic lipase, lipoprotein lipase (LPL), endothelial lipase, phosphatidyl serine phospholipase A1, and lipase H.

Most HL is synthesized and secreted by liver parenchymal cells, and then localizes mainly at the surface of hepatocytes (in the Space of Disse) and hepatic vascular endothelial cells (1), where it binds to heparan sulfate proteoglycans (HSPGs). HL is active mainly in the liver, but also in the adrenals and ovaries (2). Recently, mouse and human macrophages were identified as a site of *de novo* synthesis of HL. In mouse peritoneal macrophages, RNA levels reached 10-30% of that in liver (3).

HL displays both phospholipase A1 and mono-triglyceride hydrolase activities and hydrolyses fatty acids at the sn-1 position in both phospholipids and mono/di/tri-glycerides (4). HL is a serine-protease which hydrolyzes its substrates utilizing a single conserved Ser-Asp-His catalytic triad (5;6).

Seventy per cent of the secreted lipase activity in *in vitro* assays was found to be sensitive to immuno-inhibition by an anti-hHL antibody. Meanwhile, only 20% of intracellular activity was immuno-inhibited, suggesting that HL represents only a small fraction of intracellular lipase activity in *in vitro* assays (7). This finding points out to the importance of having a specific assay for assessing HL mass in order to reliably quantify HL levels.

Human HL (hHL) is a 476 amino acid glycoprotein with an NH₂-terminal domain, a short spanning region, and a COOH-terminal domain. The human enzyme has four N-linked carbohydrates of the complex type (8;9), with the glycosylated form of the hHL monomer running at around 65kDa on SDS-PAGE (10). The presumed active homodimer would have a head to tail orientation (11), with the COOH-terminal domain of one protomer (containing the substrate entry site) facing the NH₂-terminal catalytic domain of the other protomer. Rat hepatic lipase (rHL) has only two N-linked oligosaccharides with an apparent monomeric molecular weight 57kDa.

Heparin in the cell culture medium increases the release of HL activity in HepG2 cells in a linear fashion for 6-12 hours, time after which the medium activity starts to decrease again. Low concentration of heparin (10U/mL) leads to a release of ~70% of

HL activity into the medium, while optimal release is observed at a higher level of 200U/mL (7;12).

After two hours of heparin treatment, the observed increase in HL secretion would at least partly be due to a decrease in HL degradation with no change in synthesis rate (13). In line with this idea, optimal clearance of HL from the plasma requires both HSPGs and the low density lipoprotein receptor (LDLR) related protein (LRP). Some internalization of HL exists in LRP-deficient cells but HSPGs are required for internalization/degradation to occur (14). Heparin competes with HSPGs for binding to HL. Further, the initial binding of HL to HSPGs is a prerequisite before HL can be transferred to the LRP and subsequently internalized. Thus, heparin prevents HL binding to both HSPGs and the LRP and strongly inhibits subsequent HL clearance.

Overall, during the first ten hours, heparin increases HL secretion without a change in synthesis. Over longer incubation times, rHL synthesis may also be increased (15;16).

The use of inhibitors of the glycosylation pathway (17;18) and site-directed mutagenesis (19;20) has shown that complete N-linked glycosylation is a pre-requisite for the synthesis and secretion of fully active HL. Upon reaching maturity in the endoplasmic reticulum (ER), functional HL would be rapidly glycosylated in the Golgi and directly secreted (21) with a half-life in the cell of about an hour.

1.1.2 Functions of HL in metabolism

For an overview of the main roles of HL in lipoprotein metabolism, see Figure 1-1.

By hydrolyzing phospholipids (PL) and triglycerides (TG) in chylomicron remnants, HL exposes apolipoprotein determinants such as apoE (14) that target these particles for removal by hepatic receptors (22). The use of heat-inactivated lipase, anti-HL antibodies, or expression of catalytically inactive HL protein (HL145G) has shown that HL-mediated changes in plasma lipid profile are not all attributable to its lipolytic function. HL functions as a ligand to “bridge” lipoproteins and their hepatic

receptors for the uptake of TG-rich lipoproteins by cell surface receptors and HSPGs (23). Its combined role as a lipolytic enzyme and as a ligand increases both the hepatic clearance of chylomicron remnants (24-26) and other apo-B containing lipoproteins, as well as the scavenger receptor class B type 1 (SR-B1)-mediated (27) selective uptake of high density lipoprotein-cholesterol esters (HDL-CE) (28;29) in the liver. HL also increases the uptake of HDL-CE in steroidogenic tissues (such as adrenals and ovaries) where SR-B1 is also expressed.

One human study compared the lipid profiles of three patients with complete HL deficiency, without circulating activity but with or without a detectable (20% of normal) level of HL mass. The results suggested that inactive HL protein affects very low density lipoprotein (VLDL) and intermediate density lipoprotein (IDL) concentrations, as well as HDL concentration to a lesser extent, while HL activity influences the TG content of all lipoproteins across the density range (30). Similarly, in HL knockout (-/-) apo E^{-/-} mice, liver-specific expression of inactive HL (HL145G) lowers apoB-containing lipoprotein levels but only the wild type mice had decreased HDL levels, showing that quantitatively, the ligand bridging function of HL is mainly important for the clearance of apoB-containing particles. Note that the clearance role of HL would probably not have been unmasked without the apoE deficiency in this model (31).

Overall, by increasing the chylomicron remnant clearance rate and the selective uptake of HDL-CE, HL increases cholesterol uptake by cells. Accordingly, disruption of the HL gene in mice (or *in vivo* inhibition of HL by antibodies) results in a depletion of female adrenal cholesterol stores. Modification of low density lipoprotein (LDL) by HL can increase the uptake and accumulation of LDL cholesterol in cultured macrophages (32). Of interest, it was recently reported that in peritoneal macrophages isolated from HL-expressing apoE^{-/-} mice (as compared with HL^{-/-} apoE^{-/-} mice), the uptake of CE from oxidized LDL, and the subsequent degradation of oxidized

LDL were increased (33). Given that HL is now known to be expressed in macrophages from aortic lesions, this could have profound consequences in accelerating the cholesterol loading and thus the development of lesions. The presence of HL in the arterial wall could prove to play a similar role as that which has been described for LPL, locally increasing the free fatty acid (FFA) concentration, causing increased and prolonged retention of LDL, and increasing cholesterol uptake and foam cell formation, which would all lead to increased risk for atherosclerosis.

HL activity is inversely correlated with HDL-cholesterol (HDL-C) levels. Mutations resulting in a decrease in HL activity are rare, but so far they have all been associated with elevated HDL-C levels and higher risk for cardiovascular disease (CVD) (34;35). Studies on mice (36) or rabbit (37) transgenic models and HL-/- mice (38) clearly show that HL plays a major role in determining HDL-C levels. HL overexpression is associated (37) with a marked decrease in HDL-C and decreases in HDL, HDL-apoA1, HDL-apoCIII, and HDL-apoE. Androgen administration in men also leads to an increase in HL activity which precedes and is highly correlated with a decrease in HDL-C (39).

The hydrolysis of the TG in the core of the HDL₂ particle and of PL by HL is coupled with a dissociation of apoA1 and a decrease in the HDL₂ particle size into smaller, denser HDL₃ (40). The HDL₃ particles are cleared more rapidly from plasma because they are optimal substrates for selective uptake (they are either taken up by the liver or excreted by the kidneys). apoA1 can re-associate with phospholipids and cholesterol to generate pre β 1-HDL lipid complexes (41). These pre β 1-HDL particles, which are smaller than HDL₂, are good acceptors of effluxed cell cholesterol and originate reverse cholesterol transport (42).

HL hydrolyzes IDL (products of the hydrolysis of VLDL by LPL) into LDL (43). In patients with HL deficiency, β -VLDL particles (rich in cholesterol) as well as IDL particles accumulate and LDL levels are low.

The atherogenicity of LDL is linked to the levels of LDL-C, but also to LDL size and density. Small, dense LDL particles are associated with increased risk for CVD and with high HL activity, which is a key enzyme involved in their formation. Some of the effects of lipid-lowering therapy that are not mediated by a decrease in LDL-C could be due to an HL-mediated change in LDL buoyancy (44).

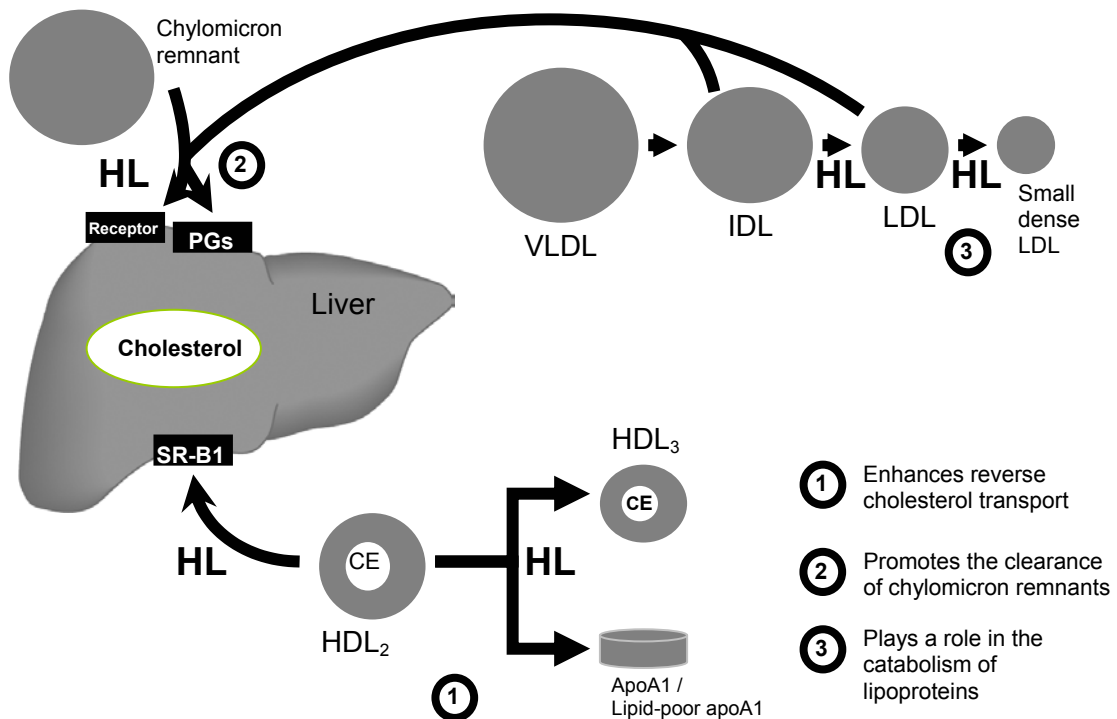


Figure 1-1: Main roles of hepatic lipase (HL) in lipoprotein metabolism

1.1.3 Is HL pro-atherogenic or anti-atherogenic?

HL has been shown to have proatherogenic effects:

- 1) There is an inverse relationship between HL activity and HDL-C concentrations. Low levels of HDL-C are in turn positively correlated with higher risk for atherosclerosis.
- 2) There is a small positive correlation between small LDL and HL activity which hydrolyzes IDL into LDL (43).

- 3) apoE ^{-/-} HL^{-/-} mice have less atherosclerosis than apoE^{-/-} only mice (45), suggesting that mHL (which is mostly circulating) is pro-atherogenic in this model.
- 4) Bone marrow transplantation experiments showed that even though macrophage HL expression does not affect the lipid profile and lipid content of lipoproteins, macrophage HL enhances early lesion formation in apoE^{-/-} HL^{-/-} mice (33). In these experiments, apoE^{-/-} or apoE^{-/-} HL^{-/-} mice were subjected to total body irradiation before being injected fetal liver cells of either genotype as a source of hematopoietic cells. Macrophage expression of HL in apoE^{-/-} HL^{-/-} mice raised lesion size to levels similar to that seen in apoE ^{-/-} mice which also express HL in liver and other tissues, indicating that macrophage HL specifically has a major role in aortic lesion formation in mice.
- 5) The fact that HL-deficient macrophages have reduced aortic atherogenesis was confirmed by similar bone marrow transplantation experiments in the lecithin: cholesterol acyltransferase (LCAT) transgenic model, which is independent of apoE deficiency (33).

On the other hand, HL also has been shown to have several antiatherogenic effects:

- 1) Hypertriglyceridemic mice overexpressing human HL have less atherosclerosis (36).
- 2) Liver-specific overexpression of hHL (mostly bound to cell surfaces) in LDLR^{-/-} HL^{-/-} mice reduces atherosclerosis, and this requires the lipase activity since similar expression levels of catalytically inactive human enzyme does not alter atherosclerosis rate in this model (46).
- 3) During post-prandial lipid clearing, HL favors the clearance of chylomicron remnants, which are known to be highly atherogenic.
- 4) Expression of inactive HL (HL145G) in the liver of apoE^{-/-} HL^{-/-} mice is (in contrast to wild-type mouse HL) protective against proximal aortic atherosclerosis, at

least partly because of the lowering of cholesterol-rich remnant concentration in plasma via the HL-LRP pathway (31). This identifies the ligand-bridging function as anti-atherogenic in this mouse model.

5) The role of HL as a lipolytic enzyme and as a ligand increases the selective uptake of HDL-CE in the liver (28;29) mediated by the SR-B1 (27).

6) HL increases formation of pre β -HDL (of discoidal shape). Thus at several levels (5 and 6), HL is implicated in reverse cholesterol transport (RCT).

7) HL deficiency is a rare lipid disorder that has been identified in only 5 families (39;47-50). The lipid abnormalities associated with this disease include elevated IDL, with buoyant HDL and LDL containing abnormally high levels of TG and PL (34;51). There is low LDL and accumulation of cholesterol-rich IDL and β -VLDL in plasma (consistent for a role of HL in clearance of VLDL and chylomicron remnants, and conversion of IDL to LDL). HL-deficient patients have hypertriglyceridemia (across the lipoprotein density spectrum), hypercholesterolemia, and premature atherosclerosis (47)(39).

8) Low HL has been shown to be an independent risk factor for atherosclerosis (52).

Overall, as Hans Jansen concluded, “HL, is it a friend or foe? [It] seems to depend on whether it is in good or bad company” (53). The site of expression (liver vs. macrophages), the circulating levels of small dense LDL or other components of the genetic background (e.g. LDLR, ATP-binding cassette transporter A1 (ABCA1), cholesterol ester transfer protein (CETP)) may be crucial determinants whether high HL can be beneficial or not. Low LDLR activity or low CETP, which would decrease the clearance of cholesterol through apoB-containing particles, may make HL more necessary to support the RCT pathway. An HL-mediated increase in RCT, which is antiatherogenic, may outweigh the proatherogenic effect of LDL hydrolysis to smaller denser LDL if the LDL levels are low to start with.

1.1.4 Regulation of hepatic lipase

Even though HL is very similar to LPL, regulation of these two enzymes appears to be markedly different. LPL localizes to adipose tissue, skeletal muscle and macrophages, whereas HL is present in liver, steroidogenic tissues and macrophages.

Because of its implications in CVD risk, the study of HL regulation is timely and significant. HL activity is regulated at different levels including synthesis, secretion, degradation, transfer, and binding to endothelial cells. Multiple factors or hormones seem to affect HL such as insulin (positively in some but not all *in vitro* studies, the HL gene regulation by insulin has not yet been clearly demonstrated), cAMP, estrogens (inhibitory), thyroid hormone, androgens (inhibitory), apolipoproteins (although the exact nature of their physiological effect is unknown because in the *in vitro* assays, the effect seems to depend on the surface pressure of the lipid monolayer). HL is regulated by cholesterol and fatty acids, in particular by the level of fatty acid in the diet and their degree of saturation, but overall, the effects of foods on HL activity are not well known and little is understood with regards to the nutritional regulation of the gene. Postheparin plasma HL activity varies widely in the general human population, about 8 fold. Overall, around 40-60% of HL variability is thought to be genetically determined (54). Male gender, type 2 diabetes, high body mass index, intraabdominal fat (independently of total fat content), and the Lipc genotype (-514C haplotype, see 1.1.4.3) are all independent predictors of HL activity, and all are associated with an increase in HL activity. HL expression also varies depending on ethnicity.

Some questions regarding work on the regulation of HL have to be addressed, such as understanding whether the effects of HL regulators are indirect or direct, or whether the experimental system effectively reflects physiological conditions. The results found in different studies are not always concordant, at least partly because of heterogeneous experimental conditions and models. The outcome of clinical studies

also varies depending on multiple environmental or genetic interacting factors. Divergent results in the literature stress the complexity of HL-associated lipid metabolism.

1.1.4.1 HL gene (lipc) information

Gene structure

The human HL gene (lipc) is located on chromosome 15q21-23 (9;55) and consists of 9 exons with 8 introns spanning around 135 kilobases (kb) (NCBI# NT_010289). Within the hHL promoter region, major and minor transcription sites have been located 43 and 54 nucleotides upstream of the translation start codon (56-58). Some potential regulatory elements have been proposed by looking for consensus sequences (56;58;59) although few elements of the proximal promoter have yet been submitted to detailed analysis. The rat and human HL genes display no significant similarity upstream of hHL-383 and rHL-361. As a result, some regulatory sequences controlling expression of the lipc gene in these species may differ.

Deoxyribonuclease 1 (DNase 1) protected sites in the hHL promoter

A map of the human HL proximal promoter is presented in Appendix Q.

DNase 1 footprint analysis with deoxyribonucleic acid (DNA) fragments spanning the -483 to +129 region of hHL and rat liver nuclear extracts identified 8 protected regions, 4 upstream of the transcription start site (A, -28/-75, B-96/-106, C -118/-158, D -185/-255) and 4 in the first exon of the gene (E1 -5/+20, E2 +36/+55, E3 +58/+83, E4 +86/+107). Region A binds hepatocyte nuclear factor 1(HNF1). The region from +28 to +129 contains a functional negative regulatory element, which deletion enhances promoter activity by 17 fold. E2 would have a dominant negative role while E3/E4 would be required for maximal repression (60).

Oka et al. (59) used sequences from -1865 to -1330 and from -643 to -48 in the hHL promoter and nuclear extracts from HepG2 and HeLa cells to identify seven protected regions: A (-1540/-1527), B(-1505/-1473), C(-1467/-1460), D(-595/-577),

E(-565/-545), F(-234/-220), F(-234/-220) and G (-70/-48). Promoter activity analyses allowed them to identify major negative regulatory elements -1576/-1532, -623/-407 (in HepG2 cells mainly), -138/-50 (in HeLa cells mostly), as well as positive regulatory elements (-50/-9 (in HepG2 cells), -407/-138 (HeLa cells), and -1862/-1576 (HepG2 cells). Thus, footprints A-E lie in regions of negative regulatory elements.

Proposed role of HNF1 in HL gene expression

Chang et al. (58) have shown that the motif for binding of transcription factor hepatocyte nuclear factor (HNF) 1 at rHL-58/-44 or hHL-67/-53 is a functional binding site, which binds to HNF1 *in vitro* and increases the activity of a heterologous promoter in primary hepatocytes. However, in chloramphenicol acetyltransferase (CAT) transfection assays, the HNF1 site alone was not sufficient to induce activity in the context of the 3.4 kb hHL promoter in a liver-derived HepG2 cell line. Sequences from +1 to +27 in hHL were essential for basal promoter activity, while some negative regulatory elements were located upstream of hHL-222. Some regulatory sequences must lie outside of the 3.4kb promoter.

A mouse model knockout for HNF1 α (Tcf1^{-/-} mice) has been developed and analyzed using microarray technology (61). In this model, there is an increase in circulating HDL concomitant to an increase in LCAT and a 3.4 fold decrease in HL expression. Whether HNF1 α was directly responsible for the downregulation of HL was unresolved. Some other transcription factors were affected by the deficiency in HNF1 α , including an increase in sterol regulatory element binding protein 1 (SREBP1) (1.7 fold) and peroxisome proliferator-activated receptor γ (PPAR γ) (6 fold), and a decrease in HNF-3 α (2.2 fold) and farnesoid X receptor (FXR) (2 fold). Also, liver free cholesterol levels were 14% lower in the knockout mice as compared with control mice.

1.1.4.2 HL regulation by cholesterol

Because high cholesterol levels are associated with increased risk for CVD, and HL is implicated in cholesterol metabolism at multiple levels, the issue of HL regulation by cholesterol is of particular interest, although little is known to date about the mechanisms of such a regulation. Although several studies have looked at postheparin plasma levels of HL upon feeding a high cholesterol diet in various animal models, results varied greatly depending on the model, diet, and experimental procedures used. Also, measurement of postheparin plasma HL is non-specific, and therefore not always reliable by itself. Very few studies have looked at a more simplified model in tissue culture.

Since HL can affect cell cholesterol levels, in part by increasing cholesterol uptake, an attractive hypothesis is that some metabolites in the cholesterol pathway (synthesis and degradation) could regulate HL. Yet, to date, no sterol response elements have been identified to be functional in the HL promoter. Of note, LPL is downregulated by sterols, but contrary to HL, LPL is activated by SREBP through binding to a sterol responsive element (SRE) in the proximal promoter (62).

In vivo cholesterol feeding studies

Cholesterol-feeding was found long ago to increase both cholesterol ester transfer protein and HL activity in rabbits (63;64). HL activity increased three fold upon feeding rabbits with cholesterol for two days, and remained high in heparin eluates of liver tissues or in post-heparin plasma over a month of feeding. This increase in activity was not paralleled by an increase in liver mRNA levels during the first week, and HL mRNA was only increased by 46% on day 28 of treatment (65). The increase in chylomicron remnants and β -VLDL upon cholesterol feeding would induce or stabilize HL activity within 4 hours with no change in mRNA levels (66). In Rhesus monkeys (67), post-heparin HL activity increased during the first 21 days of cholesterol feeding, then decreased to basal levels when plasma cholesterol

accumulation began. However, this increase in HL activity following a cholesterol-rich diet seems to be species-specific. Early reports in guinea pigs were inconclusive since cholesterol-fed guinea pigs showed either depressed postheparin plasma HL activity (68) or doubled HL activity in hepatic heparin-releasable eluates (69). In rats, 0.5-2% cholesterol-enriched diets consistently lead to a decrease in HL activity and/or expression. A 15 to 23% decrease in postheparin plasma hepatic HL activity was found in hypercholesterolemic RICO rats (model with increased cholesterol biosynthesis in liver) (70) and normocholesterolemic Wistar rats (71). A decrease in HL activity was also found in rat liver homogenates (34%) from female lean Zucker rats (good responders to cholesterol) and this was paralleled by a similar 34% decrease in HL mRNA in liver (72).

One study determined the effect of cholesterol feeding on the hepatic mRNA expression of various transcription factors involved in cholesterol metabolism (73). Relative to a chow diet standard, chow + 2% cholesterol decreased sterol regulatory element binding protein (SREBP) 2 levels, but strongly increased SREBP1 levels.

Tissue culture models

Perhaps the best designed tissue culture study was that of Busch et al. (74), who studied the synthesis and expression of HL in response to changes in cholesterol biosynthesis in HepG2 cells, and suggested an inverse correlation between cell cholesterol content and HL mRNA/activity in these hepatoma cells. All experiments in the Busch study were done in lipoprotein-deficient serum-containing medium, depriving the cells of an exogenous source of cholesterol. Other papers followed up on the Busch study and found concordant or confounding results. Below is a summary of the effects of treatments which interfere with intracellular sterol metabolism on hepatic lipase regulation.

- Lipoprotein-deficient serum (LPDS)

LPDS, which reduces intracellular cholesterol availability, was consistently found to increase HL secreted activity after 24 (74) or 48 hours (75) by up to 50%. Longer incubation times in LPDS medium resulted in decreased secreted activity in the medium, suggesting that some degradation is taking place (74). One group found a simultaneous increase in HL mRNA and secreted activity, indicating a regulation at the transcriptional level (15).

- Statins

Statins, such as mevinolin or compactin, are inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the enzyme catalyzing the rate-limiting step in cholesterol biosynthesis.

After a 48-hour pretreatment in LPDS, Busch et al. treated the cells with 37 μ M mevinolin in LPDS medium. In such conditions, the cells were deprived of exogenous lipids and cholesterol biosynthesis was blocked. It is now known that under such conditions the sterol regulatory element binding protein isoforms SREBP2 and SREBP1a would have been strongly upregulated, whereas SREBP1c would have been downregulated. After 24 and 48 hours, HL secreted activity was increased by 2.2 and 4.9 fold respectively compared to LPDS medium control and this was paralleled by an increase in HL mass. After 48 hours, HL mRNA levels were increased by 1.8 fold. Without pretreatment, the maximal induction of HL mRNA and activity was observed after 24 hour incubation (74).

In contrast, compactin was found to decrease HL activity in HepG2 cells in other studies. In one study, HepG2 cells in RPMI medium without serum were treated for 48 hours with 20 μ M compactin. The medium was changed and incubated 3 hours with 200U/mL heparin for collection. Cell cholesterol and HL secreted activity decreased by 30% and 56% respectively, whereas LDLR activity increased by 122% (12). In another study, 10 μ M atorvastatin (Pfizer) led to a reproducible yet not

significant trend towards a decrease in secreted HL activity and mRNA levels and -685/+13-CAT promoter activity (76).

- Mevalonic acid

After a 48 hour pretreatment in LPDS medium, treatment with 1mM mevalonic acid (the product of the HMG-CoA reductase reaction, an intermediate in the cholesterol biosynthesis pathway) alone or with 37 μ M mevinolin for 48 hours resulted in a 53-65% decrease in secreted HL activity respectively (compared with LPDS medium control) without affecting HL mRNA levels. Cell cholesterol increased by 26 and 42% respectively. Mevalonic acid inhibited the induction of HL expression (74).

- LDL

In the Busch paper, 30 μ g LDL protein/mL or 100 μ g HDL protein/mL alone in LPDS medium had no consistent effect on HL. However, after a 48 hour preincubation in LPDS medium, a 48-hour treatment with mevinolin and LDL further induced secreted HL by 43% compared to mevinolin alone, with no change in mRNA levels. This synergistic effect was not yet apparent after 24 hour treatment only (74).

A few groups have reported that LDL could down-regulate HL secretion. HepG2 cells were preincubated for 48 hours with 200 μ g/mL LDL in RPMI-1640 before the medium was changed and collected after 3 hours with heparin. Addition of LDL led to a moderate (25%) increase in cell cholesterol and a marked reduction in LDLR and secreted HL activities (12). When administered in 5% fetal bovine serum (FBS) or complete medium, human LDL decreased HL activity in a dose-dependent fashion after 48 or 36 hours (with addition of heparin 24 hours within the treatment period) down to 10% (75) or 30% (77) of control respectively at 1mM LDL-cholesterol. Simultaneously intracellular stocks of unesterified/free cholesterol were increased whereas cholesterol biosynthesis was decreased. However these results were not consistently found in other studies and the authors did not raise the problem of cell toxicity that arises from adding LDL to serum-containing medium.

- 25-hydroxycholesterol

After a 48 hour pretreatment in LPDS medium, exposure of cells to 25-hydroxycholesterol (an oxidized metabolite of cholesterol) for 24 hours produced a small but reproducible induction of secreted lipolytic activity with no change in mRNA levels. 25-OH-cholesterol in combination with mevinolin strongly repressed HMG-CoA reductase and resulted in a further induction of secreted HL activity and mRNA than with mevinolin alone (74).

- Bile salts

Bile salts such as chenodeoxycholic acid (CDCA) are synthesized from cholesterol. CDCA decreases HL activity (75) and HL mRNA in a dose-dependent manner in HepG2 cells and in primary human hepatocytes (78), an effect which might be species-specific. A physiological level of bile acids (25 μ M CDCA) is sufficient to inhibit HL, while 1 μ M GW4064 (a non-steroidal synthetic farnesoid X receptor (FXR) agonist) has the same effect. Silencing experiments with small interfering RNAs (siRNAs) and treatments with FXR agonists showed that bile acids down-regulate HL through the FXR. In HepG2 cells, FXR/retinoid X receptor α (RXR α) overexpression in transactivation assays suggested that the bile acid responsive element lies within the -541/-698 region. There is no inverted repeat-1 (consensus FXR/RXR α binding element) in the hHL proximal promoter, and gel shift assays failed to show binding of FXR to oligos covering the -541/-698 sequence, suggesting that regulation by FXR may be indirect (78), which would also explain the delay in downregulation of HL. The possibility of a bile acid-mediated posttranslational modification is not ruled out.

- Oleic acid

Addition of oleic acid to HepG2 cells for 36 hours (with addition of heparin within 24 hours) resulted in an increase in secreted HL activity by up to 30% at 1.2mM oleic acid and a decrease in cell cholesterol concentration (77). In a more recent study, treatment of HepG2 cells for 48 hours with 1mM bovine serum albumin (BSA)-bound

oleate (1:6 ratio) resulted in a 34% increase in secreted HL activity (with heparin present over the last 12 hours), a trend towards an increase in HL mRNA levels, and a two-fold increase in the activity of a -685/+13 hHL promoter CAT reporter construct (the HL promoter sequence corresponded to the wild-type C-514, G-250 promoter variant) (76). Promoter activity of the same -685/+13 construct with the -C514T allele was significantly less stimulated by oleic acid or linoleic acid (79). Atorvastatin inhibited the oleate-induced increase in HL secretion and promoter activity. However, these promoter activity data are confounded by the fact that the normalizing vector used was upregulated by changes in SREBP as described below.

- SREBP / upstream stimulatory factor (USF)

In order to investigate a potential role of SREBP in regulating HL, a pSRE-luc construct containing three sterol responsive elements (SREs) from the HMG-CoA synthase gene in the pGL3 backbone was used. Because luciferase expression is driven by the SRE elements, this vector can be used to monitor mature SREBP transcriptional activity in the nucleus (76). The activity of this reporter construct increased in a dose-dependent fashion by up to 10 fold upon gradual substitution of the 20% fetal calf serum (FCS) in the medium by LPDS. At an intermediate 10% LPDS/10% FCS concentration used throughout the study, the pSRE-luc reporter construct was sensitive to either a decrease or an increase in mature nuclear SREBP (nSREBP) activity. In these conditions, oleate resulted in a 50% decrease and atorvastatin resulted in a 2 to 4 fold increase in SREBP activity. Co-transfection with pcDNA3-SREBP2 resulted in an increase of over 6 fold in pSRE-luc activity. The activity of a -685/+13HL promoter construct decreased by 75% in chloramphenicol acetyltransferase (CAT) assays normalized for β -galactosidase mass (as assayed by enzyme-linked immunosorbent assays (ELISAs)) while there was only a slight significant decrease when using a pGL3 basic construct and normalizing for μ g cell protein. RSV- β -galactosidase (Promega), the normalizing vector used in CAT assays,

was strongly upregulated by SREBP2. Thus, the marked decrease in hHL promoter activity found in the CAT assays probably reflected an increase in β -galactosidase mass, a problem which the authors did not address directly. Altogether, this study could not conclusively show that SREBP and HL promoter activity may be inversely regulated.

Upstream stimulatory factor USF1, like SREBP, is a basic helix-loop-helix leucine zipper transcription factor which binds E-boxes. USF1 is known to upregulate HL (80). Co-transfection with pCX-USF1 (a USF1 expression vector) did not affect endogenous SREBP activity but it increased HL promoter activity of the -685/+13-CAT by about four fold (76). Atorvastatin or nuclear SREBP2 cotransfection almost abolished the USF-mediated stimulation of HL promoter activity. Conversely oleate could not further increase HL promoter activity in USF1-transfected cells, presumably because USF levels were high enough to compete with the increased endogenous SREBP levels in the absence of oleate (76). Using the luciferase assay with no normalizing vector, at constant levels of USF1, there was a dose-responsive decrease in HL promoter activity upon increasing levels of SREBP2. Potential E-boxes were found at -514 and -310 of the hHL promoter, while a potential sterol responsive element (SRE) was located at -553. Yet, the regulation by SREBP and USF seems to be independent of all three putative sites since a -305/+13-luc construct displayed the same regulation pattern as the -685/+13 construct. Based on this, the authors concluded that SREBP would interfere with USF-mediated upregulation of the HL promoter, at least partly via a non E-box dependent mechanism (76). USF and SREBP2 could either bind to some unknown sequence within the -305/+13 promoter region, or recruit other transcription factors to the promoter. Under basal conditions, endogenous SREBP would partly inhibit USF-mediated stimulation of HL, which would allow for oleate to increase HL by decreasing mature SREBP levels (76). Such a theory could explain why omental fat mass or type 2 diabetes are associated with

high HL activity, since both conditions are associated with increased fatty acid (FA) supply to the liver. Also, the fact that atorvastatin treatment did not affect basal HL expression, but inhibited the oleate-mediated increase in HL expression could explain why lipid-lowering therapy has shown to be effective at lowering HL in hyperlipidemic patients in some studies (81;82).

In most cases, SREBP activates gene transcription. HL could be one of the few genes that are downregulated by SREBP. For a review of the downregulation mechanisms of these other genes, see 1.2.3.4.

1.1.4.3 HL regulation in the context of the HL promoter polymorphism

The HL promoter contains four polymorphisms (G-250A, C-514T, T-710C, A-763G according to the Ameis nomenclature (56)) which are all in Hardy-Weinberg equilibrium (83) and are in almost complete linkage disequilibrium (84). The mutant haplotype is therefore commonly referred to globally as the -514C/T polymorphism/haplotype. Note that earlier papers using the nomenclature of Cai et al. described it as the -480 polymorphism (57).

This minor allele is very common in the hHL promoter, and its frequency in the population varies from around 20% in Caucasians to around 50% in African and Japanese Americans.

The C-514T accounts for 38% of the variability of the catalytic activity of the enzyme (44;85), and the minor haplotype is associated with lower HL activity in both male and female human subjects (86). In mouse and human (HepG2) hepatoma cell lines, promoter/reporter transfection studies have shown that the C-514T mutation decreases transcriptional activity of the HL gene by up to 50% independently of (87) or only together with the mutation at -250 respectively (80).

At the level of the lipid profile, the -514T allele is associated with high HDL-C (84) with almost no effect of gender, race, or risk factors (88). High HDL-C reflects

mainly high levels of largeTG-enriched HDL₂ (89). Note that there is no association with the level of LDL-C.

HL promoter polymorphism and risk for CVD

Despite apparently favorable effects on the lipid profile, such as an increase in HDL-C, the -514T mutant polymorphism was associated with a marginally increased risk for atherosclerosis, as shown by studies which found a greater risk for angiographic coronary artery disease (CAD) (52) and an increased risk for ischemic heart disease, even after adjustment for HDL-C, age and gender (90), particularly in the presence of at least one apoE4 allele. Another study found that the -514T haplotype was associated with subclinical coronary heart disease in the background of type I diabetes as measured by an increase in coronary artery calcification (91). Higher HDL₂ levels could be the consequence of a reduced RCT flux (52) since low HL activity could result in lower rate of formation of nascent pre β -HDL and lower SR-B1-mediated uptake of HDL-CE in the liver. Also, there may be an effect of altered TG-rich lipoprotein remnant catabolism.

On the other hand, the -514C wild-type allele was associated with higher carotid intima-media thickness in the Northern Manhattan prospective cohort study (92), as well as with an abundance of macrophages in the carotid plaque with fewer smooth muscle cells, which are features of an unstable atherosclerotic plaque (93).

Gene-statin therapy interaction

A substantial number of patients undergoing lipid-lowering therapy for primary and secondary coronary heart disease prevention do not respond to treatment and eventually develop coronary heart disease. Zambon et al. (81) have shown that intensive cholesterol-lowering therapy in humans with an HMG-CoA reductase inhibitor or nicotinic acid in association with colestipol (a bile acid sequestrant), but not colestipol alone, lowers LDL-C as expected but also significantly decreases plasma HL activity and increases LDL buoyancy, which partly accounts for the drug-

associated decrease in CAD. For example, atorvastatin (Lipitor) in 40 patients with familial hypercholesterolemia (FH) for 12 weeks decreased HL activity by 18% and plasma cholesterol and TG (94). It is tempting to speculate that the improvement in LDL buoyancy is due to the decrease in HL activity and this would make HL a potential therapeutic target.

In the Familial Atherosclerosis Treatment Study, lipid-lowering drugs that appear to involve an HL-associated effect have been suggested to be more effective on the -514 CC genotype (44). This effect was similar whether the treatment was lovastatin and colestipol or niacin and colestipol. Homozygous -514 CC patients, which originally have more small, dense LDL particles and lower HDL-C, showed a greater decrease in HL activity, and a greater increase in LDL buoyancy than -514 CT or TT patients. Coronary stenosis improved most in -514 CC patients and to a lesser extent in CT patients, but aggravated in TT patients. Therefore, the HL polymorphism would strongly influence the outcome of clinical therapy with lipid-lowering drugs.

Another study looked at atorvastatin treatment in male and female of the Diabetes Atorvastatin Lipid Intervention study, which is a randomized, placebo-controlled multicenter study with subjects of various ethnic backgrounds and living in various countries. In this study, in the background of type 2 diabetes, atorvastatin led to a dose-dependent decrease in postheparin HL activity, but the magnitude of the effect was independent of sex or the C-514T promoter variant. Again in this case, this raises the possibility that diabetes or associated traits (such as FFA or higher waist-to-hip ratio) could interfere with the effect of atorvastatin on HL (82).

Gene-insulin responsiveness interaction, effect on USF binding

Insulin resistance is associated with increased plasma HL activity and low LDL-C in both non-insulin dependent diabetes mellitus (NIDDM) patients and non-diabetic subjects (95;96). HL is elevated in NIDDM (97) and has been implicated in mediating the decrease in HDL-C observed in these diabetic patients. Studies done in the

fructose-fed syrian golden hamster, an animal model of insulin resistance linked as expected the insulin-resistant diabetic state with increased HL activity, while insulin-sensitizing drugs such as rosiglitazone normalized HL levels (98).

The promoter mutation at -514 could alter an insulin response element and interfere with insulin responsiveness. HL post-heparin plasma activity was increased at higher insulin concentrations in -514CC homozygotes only (but not in carriers of the T allele) (86). The C-514T base pair substitution is at the center of a CA(c/t)GGG E-box. USF, a putative insulin responsive transcription factor that is expressed in the liver, was shown to bind at the -514 site by supershift assay and to induce HL transcription as shown by an increase in promoter activity (80). The -514C/T substitution in the HL promoter decreases binding affinity of USF to this site by around 2 to 4 fold (as shown by gel shift assays for HL specific oligos). USF may bind to the HL promoter at the -514 site or at other USF consensus present in the -685/+13 promoter fragment and lead to an increase in transcription of HL. When mutated along with -250 G/A (but not alone in this particular study), the -514C/T decreased promoter activity of a -685/+13 construct by about half in HepG2 cells, suggesting that it may play a role in basal transcriptional rate.

The mutated G-250A haplotype appears to put carriers at risk for insulin resistance in most populations (99;100). Low HL activities associated with the mutant promoter variant are associated with elevated TG, which could be the cause for conversion to diabetes. On the other hand, the G-250A mutation tends to be protective for subjects from the Finnish population (101). The reasons for this specificity remain elusive, but dietary habits such as total fat and saturated fat consumption may be involved (102).

1.2 Tools to study cholesterol regulation

1.2.1 Methyl-beta-cyclodextrins complexed to cholesterol

Methyl- β -cyclodextrins (Sigma C-4555) are cyclic oligosaccharides that can encapsulate insoluble hydrophobic compounds such as cholesterol, rendering them

soluble in an aqueous phase. They can be used to manipulate the cellular cholesterol content (103). Alone in solution, methyl- β -cyclodextrin (m β -CD) at high concentrations (10-100mM) can efficiently stimulate efflux of cholesterol and deplete intracellular cholesterol stocks from a variety of cultured cells much more efficiently than physiological concentrations of HDL. Release of cholesterol from cells occurs quickly, mostly during the first two hours (103). On the other hand, when complexed with cholesterol, cyclodextrins can be used to load cells with cholesterol (104). After treatment with 5mM CD, 8:1 cholesterol molar ratio, there is a 2.5 fold enrichment in Fu5AH cell cholesterol after a seven hour incubation (103). Alteration of cellular cholesterol levels using cyclodextrins has been used in several studies, including a study of the regulation of CD36 in macrophages (105), of SR-B1 in adrenal cells (106), and of ABCA1 in mouse aortic smooth muscle cells (107).

1.2.2 Acyl Coenzyme A: Cholesterol Acyltransferase inhibitors

1.2.2.1 Acyl Coenzyme A: Cholesterol Acyltransferase (ACAT)

Mechanistically, ACAT transfers the fatty acyl moiety of a fatty acyl CoA to the hydroxyl group of a sterol which lies embedded in a lipid bilayer (membrane). ACAT activity is present in a variety of tissues including the intestinal mucosa, the liver (Kupffer cells and hepatocytes), adrenals, testes, and macrophages (with an increase in ACAT activity during plaque formation) (108). ACAT is stimulated when cellular free cholesterol (FC) levels (its substrate) surpass a threshold above normal cell cholesterol concentrations (109), and this may be due to an FC-induced rise in ER cholesterol. ACAT plays a role in the storage of cholesterol, by esterifying cholesterol to cholesterol esters, which accumulate for example in macrophages to form foam cells, or in steroidogenic tissues as a substrate for steroidogenesis.

There are 2 different ACAT isoforms with different tissue localizations. The ACAT1 isoform has a more ubiquitous expression pattern, and in humans, it is the major isoform in liver and macrophage-derived foam cells (108). ACAT2 is the

predominant isoform in human intestine and is present at low levels in human hepatocytes. This is in contrast with other mammals such as mice and non-human primates where ACAT2 is the predominant isoform in liver. Within the cell, most of ACAT (isoforms 1 and 2) is located in the ER, with a small portion of immunoreactivity in macrophages localizing close to the trans-Golgi network or with the plasma membrane fraction (110).

Hydropathy plots suggest that both ACATs contain multiple transmembrane domains, and it is possible that the active site for ACAT1 would locate in the cytosol while the active site for ACAT2 would locate to the lumen of the ER (111;112).

1.2.2.2 Use of ACAT inhibitors

Current inhibitors of ACAT (fatty acid amides, urea based compounds, compounds with increased water solubility) are nonselective for either isoforms since they were selected before the existence of two isoforms was known. Among these, two common ACAT inhibitors are CP-113 818 (Pfizer) and 58-035 (Dr. Thomas Hughes, Novartis). 58-035 is a 3-[decyldimethylsilyl]-N-[2-(4-methylphenyl)-1-phenylethyl]propanamide compound also called PFK058-035. The IC₅₀ of 58-035 was found to be of 0.38 \pm 0.12 μ M in rat liver microsomal ACAT assays (113). It is assumed to be a direct competitive inhibitor of ACAT (114).

Commonly, during the time frame of a typical cell culture experiment, upon cholesterol loading in the absence of ACAT inhibition, cells would mainly have an increase in CE.

In cell culture (both hepatoma Fu5AH cells and cultured arterial SMCs or macrophages), when used at concentrations >0.1 μ g/mL (and up to 10 μ g/mL) in combination with cholesterol-loading agents, 58-035 selectively and effectively inhibits the esterification of cholesterol (>95%), and thus it inhibits the accumulation of CE (115). It also leads to a slight increase in cellular FC. Thus, the presence of an ACAT inhibitor speeds up the FC loading, while overall, total cholesterol in the cell

decreases (115). The inhibition of CE formation is dose-dependent and very rapid as full inhibition is observed after only 30 minutes of exposure to the cells (115). Unless cholesterol acceptors are present in the medium, ACAT inhibitors can cause cholesterol-loaded cells to undergo apoptosis and necrosis.

1.2.3 Sterol regulatory element binding proteins (SREBPs)

1.2.3.1 Different SREBPs with different roles in metabolism

There are two different SREBP genes that express SREBP1 and SREBP2 respectively. SREBP1 has two promoters, each encoding an mRNA with a different first exon (longer for 1a than for 1c, which has a shorter transactivation domain).

The combined levels of SREBP1a and 1c within the cell are generally comparable to the levels of SREBP2 (116). SREBP1c is the major isoform of SREBP1 in liver and adipose tissue, with approximately 10 times more 1c than 1a in animal liver cells. The same trend is found in McA-RH7777 (ATCC# CRL-1601) or FTO-2B cells lines (117), while non hepatic cells and most cancer cells often have more 1a (e.g. mouse NIH-3T3, HEK-293, CHO or HepG2) (118).

In transgenic mice that overexpress truncated, mature forms of SREBP1a, 1c or 2 in the liver, and in SREBP1 knockout studies, SREBP2 favors the synthesis of cholesterol from acetyl CoA, whereas the 1c isoform favors the synthesis of TG and PL. In other words, SREBP1 isoforms are more selective for activating genes involved in fatty acid biosynthesis (lipogenic genes) and SREBP2 is more specific for controlling genes in cholesterol biosynthesis (119).

Livers of mice overexpressing SREBP1a were massively enlarged, owing to accumulation of TG and cholesterol (119). Livers of mice overexpressing SREBP1c (with comparable SREBP overexpression) were only slightly enlarged, with only a moderate increase in TG but not cholesterol (120). The cholesterol-related mRNAs were not elevated, and only slight elevations were seen for mRNAs involved in FA

synthesis. Neither SREBP1a nor 1c transgenic mice displayed an elevation of HL mRNA levels (120).

Luciferase reporter gene assays in HepG2 cells showed that all three SREBP wild type isoforms activate the SRE, SRE-like, or E-box sequences in target genes, but with different efficiencies. SREBP1a was found to be a more potent activator for all gene targets (121), while the activation potential of SREBP1c was significantly weaker than that of 1a (with an extra acidic activation domain at its N-terminal) or of 2 (122). 1a and 2 can activate cholesterologenic genes (classic SREs) to almost similar extent, but 1c is very weak. 1c and 2 (the major isoforms in the liver) displayed similar activities in activating lipogenic genes. 1a and 1c can activate E-boxes, but 2 is inactive although it can bind.

Table 1-1: Relative transcriptional activities of the SREBP family (121)

	Nuclear SREBP2	Nuclear SREBP1c	Nuclear SREBP1a
SRE	+++	~0	+++
SRE-like	++	++	+++
E-box	0	+	++

Transcriptional activation of genes by SREBP is dependent on the interaction of SREBP with additional transcription factors (Sp1, NF-Y) and/or coactivators (CREB), especially for genes activated through SRE or SRE-like sequences. These interactions can stabilize the DNA complexes. NFY or Sp1 may not be crucial for E-box-mediated activation by SREBP1, which instead may be modulated by adjacent AP-1 sites.

1.2.3.2 Regulation of the various SREBPs

SREBPs are synthesized as ER-bound precursors, where they bind to the sterol-sensing domain of the SREBP cleavage-activating protein (SCAP). Cholesterol

interacts with the sterol-sensing domain of SCAP and produces a conformational change in SCAP, which triggers binding of the Insig protein and prevents the SCAP/SREBP/Insig complex from reaching the Golgi (123). When intracellular cholesterol concentrations are low, SCAP escorts SREBP from the ER to the Golgi (116), where SREBP undergoes proteolytic cleavage. Site 1 protease (a membrane-bound protease) first cleaves within the hydrophilic loop of SREBP. Then, site 2 protease (a zinc metalloprotease) cleaves within the membrane-spanning domain of SREBP, releasing the N-terminal fragment, which can translocate to the nucleus to activate transcription. 25-hydroxy-cholesterol is even more effective than cholesterol at downregulating SREBP processing. Sterols alone can reduce SREBP2 in a sustainable manner, but the ability of sterols to suppress SREBP1 (cleavage) is dependent on the presence of fatty acids (124).

A liver X receptor (LXR) binding site was identified within the SREBP1c promoter (125) which mediates the LXR-mediated stimulation of the SREBP1c isoform (126;127). Basal transcription and expression of SREBP1c specifically requires LXR and its endogenous ligands (117), which are oxysterol intermediates of the cholesterol biosynthetic pathway.

In comparison with the 1a isoform which displays low constitutive expression (non-regulated), the 1c isoform is not only regulated at the level of sterol-dependent cleavage but is also highly regulated by LXR and insulin. Thus, SREBP1c is not regulated by sterols as tightly as 1a and 2 in hamsters (128) or in liver cell lines (129). In the presence of cholesterol, LXR activates SREBP1c transcription, and as a result there can be active 1c in the nucleus at high cholesterol levels. How the SREBP1c precursor is processed to a mature transcription factor in the presence of sterols is still unknown.

In mice, SREBP1c (but not 1a or 2) is known to be induced at the transcriptional level and at the protein level in lipogenic states (high carbohydrate diet, refeeding)

(130). Lipogenic gene transcription is inhibited by polyunsaturated fatty acids (PUFA) through a decrease in nuclear SREBP1 (nSREBP1) without affecting nuclear SREBP2 (131;132). PUFA inhibition of nSREBP1 is effective in the absence of sterols whether cells are cultured in LPDS or delipidated serum medium (124). Insulin increases 1c mRNA in mice (133) and in isolated hepatocytes. Stimulation by insulin is blocked by PUFA in rat hepatocytes (134). PUFA decrease the abundance of nuclear SREBP1a and 1c by at least three mechanisms. First, PUFAs inhibit SREBP1c gene transcription specifically, with very little effect on 1a and none on 2 (135-137) in part by antagonizing oxysterol binding to the LXR and thus LXR activation (136). Cholesterol was shown to override this repression in mice fed cholesterol and PUFA (138). Second, PUFAs accelerate SREBP1c mRNA decay *in vivo* or in primary hepatocytes (139). Third, PUFAs inhibit processing of SREBP1 precursor to its mature form (135). This PUFA inhibition overrides the cholesterol-induced activation of SREBP1 protein maturation (138).

1.2.3.3 SREBP-activated genes and SREBP-binding sequences

Many cholesterol biosynthetic genes are activated by SREBPs, including HMG CoA synthase, HMG-CoA reductase, farnesyl-diphosphate synthase, squalene synthase, SREBP2, and the LDLR. Cholesterol biosynthetic genes either have the classic SRE1 (ATCACCCAC) (140) or its modified SRE3 (CTCACACGAG), and adjacent cofactors (NF-Y, Sp1) in their promoters. Mutation of nine out of the ten nucleotides of the SRE1 in the promoter of the LDLR impairs the response to sterols. The SRE is necessary for high transcription of reporter genes in sterol-deprived cells but is not required for basal sterol-repressed transcription.

A number of lipogenic enzyme genes are also under the control of SREBPs, such as acetyl CoA carboxylase, fatty acid synthase (FAS), stearoyl CoA desaturase1 and 2, glycerol-3-phosphate acyltransferase, and Spot 14. For these genes, SREBP binding and activation sites are more diverse. Some genes such as Spot 14 (141) and FAS

(142) contain E-boxes which are thought to confer sensitivity to PUFA, glucose or insulin through SREBPs. E-boxes or E-box like sequences (5'-CANNTG-3') bind members of the basic helix-loop-helix (bHLH) family such as USF1/2 (methyl sensitive transcription factors with consensus sequence 5'CACGTG3'), cmyc, inhibitors of DNA binding (Ids), SREBP1, as well as some non-members. Within the SREBP family, SREBP1 has the unique property of binding/activating E-boxes or E-box like sequences, although binding to a complete CACGTG site does not necessarily mediate activation. Maximal activation of an E-box requires SREBP concentrations up to 100 fold higher than for maximal activation of an SRE. Other genes could utilize SRE-like sequences (TCAGGCTAG) such as in ATP Citrate Lyase (143) or SRE halves (TCACCC) such as in the malic enzyme promoter (121).

Finally, other SREBP-activated genes include PPAR γ and LPL which expression is directly controlled by SREBP through an SRE site, although E-boxes are present in the promoter (62;144;145).

1.2.3.4 Genes known to be downregulated by SREBPs

More recently, SREBPs were also shown to mediate downregulation of some target genes. In all examples of downregulation by SREBP known to date as described below, genes are upregulated by sterols and downregulated by sterol depletion. For an overall view of how SREBPs can activate or inhibit transcription, see Figure 1-2.

Downregulation through binding of SREBP to SREs

Human microsomal triglyceride transfer protein (MTP) is down-regulated by SREBP through an SRE (5'-GCAGCCAC) located at -124/-116bp. A truncated form of SREBP2 lacking the transcriptional activation domain and normally acting as an inhibitor of transcription could also mediate inhibition, indicating that direct binding to the promoter itself may be sufficient. Interaction with cooperative factors seems unlikely because of the lack of Sp1 or NFY binding sites in the vicinity of the

SRE, and because of the lack of effect of deleting the transcriptional activation domain. SREBP2 could possibly act by competing for binding with AP-1, which has an adjacent binding site and is necessary for promoter activity (146).

SREBP1c downregulates PKA-stimulated (but not basal) phosphoenolpyruvate carboxykinase (GTP) (PEPCK/PEPCK-C) in isolated hepatocytes. SREBP1 or 2 bind weakly to both SREs in the promoter (-322/-313 and -590/-581), and an unknown factor is necessary for facilitating SREBP1c binding. Mutation of the -590 element only dramatically enhanced basal PEPCK expression, but a mutation of both SREs was necessary to fully relieve the promoter of SREBP-mediated inhibition. Sp1 (which has a binding site on the opposite strand of the DNA) and SREBP1 compete for binding to the -590 SRE, and displacement of Sp1 results in an inhibition of gene transcription. Both Sp1 and SREBP1 bind the promoter in this region *in vivo* as shown by chromatin immunoprecipitation (ChIP). Replacing the PEPCK SRE sequence by that of the LDLR (one nucleotide difference: ATCACCCCTC) at -590 also mutates the Sp1 binding element and allows for both SREBP1 and Sp1 to bind the consensus SRE and stimulate transcription. Replacing the -322 SRE by the LDLR SRE did not have any stimulatory effect (147).

Downregulation through binding of SREBP to E-boxes

ApoA5, a protein which associates with HDL and VLDL in plasma, is downregulated by SREBP1 in a dose-dependent manner at the promoter activity level. Two E-boxes at +10/+15 (5'-CAGGTG) and -76/-81 (5'-CACGTG) in the ApoA5 promoter bind specifically to SREBP1-c *in vitro* and are functional as shown by mutation analysis. A construct containing 3 tandem repeats of the more potent +10/+15 E-box was downregulated by SREBP1, and mutation of an SP1 site adjacent to the +10/+15 site did not affect downregulation. Overall, downregulation was conferred by binding of SREBP-1 to isolated E-boxes, with possible displacement of other bHLH-ZIP (148).

Mature SREBP2 inhibits ABCA1 promoter activity through binding to an E-box between -156 and -116. The association of SREBP2 to the vicinity of the E-box on the ABCA1 promoter was demonstrated *in vivo* doing chromatin immunoprecipitation (ChIP) analysis under sterol starvation. This E-box was previously shown to bind other transcription factors including USF1 and USF2. In this study, USF1 and 2 were shown to activate the hABCA1 promoter. USF1 levels were unchanged under sterol starvation (high SREBP levels) whereas USF2 was downregulated. SREBPs could replace USF by competing for binding to the E-box. Alternatively, SREBP2 could form a complex with other transcription factors or recruit other repressors to the promoter (149).

Downregulation without direct binding of SREBP to DNA

Sterol 12 α -hydroxylase, an enzyme required for cholic acid synthesis, is up-regulated by SREBP1 but down-regulated by SREBP2. Whereas activation by SREBP1 involves binding to two perfect inverted SREs in the proximal promoter (lower strand: 5'-TCACCCAC at -323/-330 and -306/-315), the inhibition by SREBP2 was unaffected by deletion or mutation of these SRE sites. SREBP2 repressed gene transcription apparently without binding to the promoter, possibly by binding to LRH1 (also known as FTF) thereby preventing LRH1-mediated activation of gene transcription (note: LRH1 binding may not be affected) (150).

Promoter activity of human oxysterol 7 α -hydroxylase (CYP7B1 – an enzyme active in the metabolism of oxysterols) is suppressed by SREBP1. The SREBP1-responsive region was mapped to a GC-rich region in the proximal promoter (-86 to +176) which was previously shown to bind Sp1. Three GC-boxes in this region were found to be involved in SREBP1 downregulation, and a single GC-box was sufficient to mediate inhibition. Gel-shift assays using extracts from SREBP1 or 2 overexpressing cells failed to show binding of SREBP to the promoter. SREBP1c was able to completely abolish Sp1 activation in cotransfection experiments, suggesting an

indirect mechanism whereby SREBP1 would bind to Sp1 and prevent it from binding to the GC boxes (151).

SREBP1 downregulates HNF4 α -stimulated expression of PEPCK. In this case, SREBP1 would not bind to the promoter but would bind through its transactivation domain to the ligand binding/AF2 domain of HNF4 α (as demonstrated by glutathione-S-transferase pull-down assays and *in vivo* by coimmunoprecipitation). This binding would still allow HNF4 α to bind to the glucocorticoid-response unit in the promoter but would prevent HNF4 α from recruiting the essential PPAR γ coactivator-1 and from activating transcription. A similar mechanism was hypothesized to explain the SREBP1-mediated down-regulation of glucose 6 phosphatase, another gluconeogenic HNF4 α -activated gene (152).

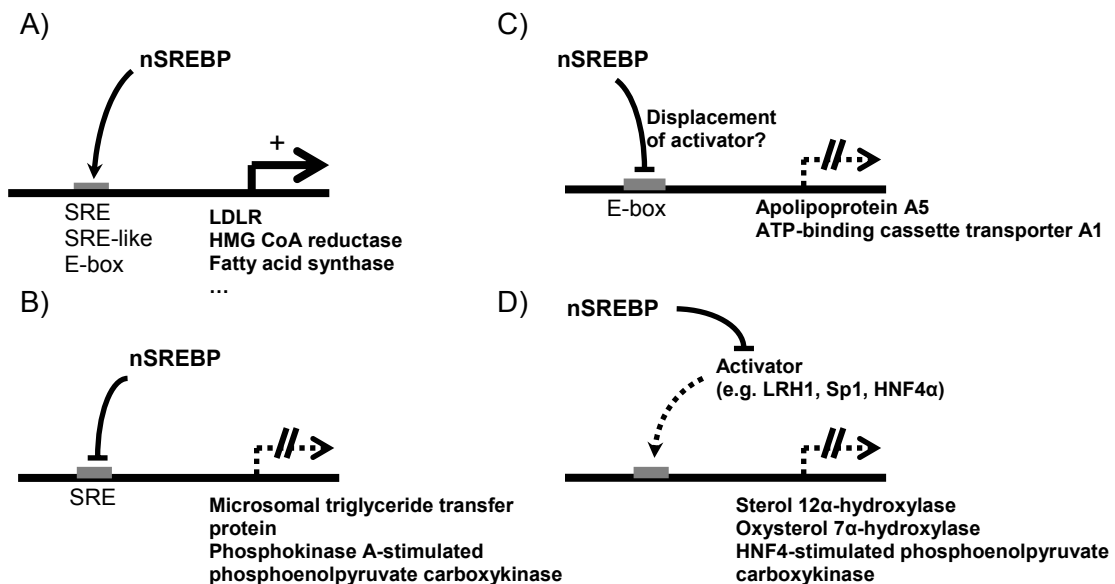


Figure 1-2: Known mechanisms for gene transcription regulation by SREBPs

SREBPs can activate gene transcription (A) through binding to sterol responsive elements (SRE), SRE-like elements, and/or E-boxes. SREBPs can also inhibit transcription of target genes through binding to an SRE (B), an E-box (C) or without binding directly to the promoter (D). Although regulation is isoform-specific for each target gene, all mature SREBP isoforms are referred to here as nuclear SREBP (nSREBP) for simplicity.

1.3 Introduction to the research chapters

This thesis investigates the regulation of HL by cholesterol and factors which affect intracellular cholesterol levels.

Chapter 2 describes the effects of cholesterol depletion and repletion on hepatic lipase protein secretion and enzyme activity levels in our tissue culture model. These data were used as a basis for all further experiments designed to investigate the underlying mechanisms for this regulation.

Chapter 3 describes the effects of sterol manipulations on HL at the level of transcription by looking at mRNA steady-state levels, mRNA degradation rate, and HL promoter activity levels. SREBPs are major transcription factors controlling the expression of genes involved in cholesterol metabolism. Thus, we also investigated their role in HL regulation using similar methods, alone or in the presence of USF1, which is a known activator of HL. Gel shift assays were performed in order to identify transcription factor binding sites within the hHL proximal promoter. Finally, co-immunoprecipitation experiments were performed to investigate a possible interaction of SREBP1 with positive effectors of HL transcription such as USF1.

Finally, Chapter 4 presents the effects of sterol manipulations at the post-transcriptional level by looking at HL protein synthesis, protein degradation, and translation efficiency.

Chapter 2 – Effects of sterol manipulations on HL protein and enzymatic activity in rat hepatoma cells

2.1 Introduction

Because of the role of HL in enhancing lipoprotein uptake and cholesterol loading, it has been speculated that HL expression could be regulated by intracellular cholesterol as an additional means to ensure feedback regulation of cell cholesterol levels (74). To date however, despite numerous studies, not much has been elucidated regarding the regulation of hepatic lipase. In particular, the regulation of hepatic lipase by cholesterol is still subject to some debate, and no underlying mechanism has been offered. Because of the apparent discrepancies between different groups regarding the effect of cholesterol treatment on HL secretion, our first goal was to clearly define the effects of cholesterol on HL protein secretion and activity levels in our cell culture model.

2.2 Methods

2.2.1 Tissue culture

Fu5AH and McA-RH7777 cells were maintained at 37°C in a 5% or 10% CO₂ atmosphere respectively in their individual growth medium. McA-RH7777 cells were grown on collagen-coated dishes in medium consisting of 20% horse serum, 5% fetal bovine serum, 2mM L-glutamine, 9.8g/L NaHCO₃, 10.6g/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 42.3g/L GIBCO Dulbecco's modified Eagle's medium #61100-103 (DMEM); pH 7.2. Fu5AH medium consisted of 10% FBS, 2mM L-glutamine, 11.7g/L NaHCO₃, 12.7g/L HEPES, 50.9g/L GIBCO #61100-103 Eagle's minimal essential medium (MEM); pH 7.2.

The cells were split upon reaching confluency using standard procedures. Briefly, the medium was aspirated and the cells were washed with phosphate-buffered saline. PBS was then aspirated and replaced by 0.25% trypsin +/- 0.1% ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS), which was removed after 30 seconds.

The cells were resuspended in medium and part of this solution was used to start a flask with new growth medium.

2.2.2 Reagents

Cholesterol (cell culture tested, Sigma C3045) and 25-OH-cholesterol (Sigma H1015) were made fresh every few days as a 500X stock (5mg/mL cholesterol, 0.5mg/mL 25-OH-cholesterol) in ethanol. Sodium mevalonate (mevalonolactone, Sigma M4667) was prepared as a 50mM stock in PBS and was stored at 4C. β -sitosterol (Sigma #S-9889) was kept at -20C as a 0.5mg/mL chloroform stock. The ACAT inhibitor 58-035 was obtained from Dr. Thomas Hughes at Novartis. It was reconstituted as a 10mg/mL stock in dimethyl sulfoxide (DMSO) and stored at -20C in aliquots. Recommended 58-035 final concentrations in cell culture range from 0.1 μ g/mL to 10 μ g/mL. Methyl- β -cyclodextrins solutions (Sigma C-4555) were prepared as described in 2.2.5.

2.2.3 Preparation of delipoproteinated serum by ultracentrifugation

Lipoprotein-deficient serum (LPDS) was made according to a protocol adapted from Busch et al. (74). FBS (of original density 1.006g/mL) was adjusted to a final density of 1.21g/mL using a 1.478g/mL solution (made with NaBr, NaCl) (153). The serum was spun for over 40 hours at 55,000 revolutions per minute (rpm), at 15C in Quick-Seal tubes (Beckman #342414) using a 60Ti rotor (Beckman ultracentrifuge), with no brake. Tubes were cut 5.1cm from the bottom, and the lower compartments were recovered on a glass cylinder on ice, making sure to rinse the sides of the tubes to resuspend and recover any aggregated protein. The delipoproteinated serum was then dialyzed overnight at 4C against 10mM potassium phosphate, 120mM NaCl, 2.7mM KCl, pH 7.4. The dialysis buffer was changed in the morning and again after a few hours. The dialyzed LPDS was recovered, adjusted to the original volume of serum using dialysis buffer, and filter-sterilized in a hood for tissue culture use. Occasionally, the osmolarity of the LPDS was checked and was consistently around 300mosm.

2.2.4 Preparation of defatted serum

In the following procedure, FBS was first treated with Cab-O-Sil to remove all lipoproteins from the plasma (154) and the remaining free fatty acids were extracted with n-butanol and isopropyl ether according to the Cham and Knowles protocol (155).

Briefly, 2% Cab-O-Sil (2g/100mL) was added to the FBS, and the serum was incubated at 4C for 4 hours with occasional gentle shaking. The serum was then centrifuged for 30 minutes at 20,000rpm using a Sorvall SS-34 rotor and the supernatant was filter-sterilized in a tissue culture hood.

To one volume serum, 1.2 and 0.8 volumes of isopropyl ether and n-butanol were added, respectively, and the mixture was incubated in a hood for 20 minutes at room temperature, followed by 20 minutes on ice, swirling regularly to ensure good mixing of the aqueous and organic phases. The mixture was then centrifuged for 10 minutes at low speed (1000rpm) in 50mL Pyrex tubes using a 921 rotor. Three phases separated, an upper organic phase, an intermediate foamy phase (which should be minimized as much as possible by spinning harder), and a lower water-soluble phase. Both upper and middle phases were aspirated out and the lower phase was submitted to centrifugation again to rid of the remaining organic phase. To ensure that there was no residual n-butanol in the water-soluble phase, 0.4 volume isopropyl ether was added to the lower phase mixture and the same protocol was followed as for the previous extraction. The final mix was flushed for 2 hours with a stream of N₂ allowing for recovery of around 1 volume of defatted serum at this step. This serum was frozen in layers by swirling the flask in a mixture of ethanol and dry ice, the shell-frozen serum was freeze-dried, resuspended in 0.2 volume with distilled water, dialyzed against PBS, adjusted back to one volume with PBS, and spun at 10,000rpm for 30 minutes in an SS-34 rotor (Sorvall) to pellet insoluble protein. Finally, the supernatant was filter-sterilized and stored at -20C in multiple aliquots. Note that this procedure affects neither ionic strength nor pH.

2.2.5 Methyl-beta-cyclodextrin (m β -CD) solutions

Methyl-beta-cyclodextrin (Sigma C-4555, MW 1317-59, average: 1338) solutions were prepared according a protocol derived from Christian et al. (103).

10mM m β -CD stock solutions in MEM were made by stirring at room temperature for 30 minutes and filter-sterilizing through a 0.2 μ m filter, and were stored at 4C up to several months in glass containers. The stock solution was diluted 5 or 2 fold respectively with LPDS, L-glutamine (2mM final) and MEM to reach 2mM or 5mM m β -CD final in the tissue culture media. The pH was checked with pH paper, and adjusted to 7.4 if necessary, although it was usually not required.

50mg/mL (129.3mM) stock solutions of cholesterol (MW: 386.67) in 1:1 chloroform:methanol were made in glass scintillation vials, sealed with Teflon lining, and flushed with N₂ after each use for up to 2-4 weeks.

To make the 5mM m β -CD 8:1 m β -CD:cholesterol molar ratio medium, cholesterol from the stock solution was pipeted into a glass round bottom flask (of adequate volume to accommodate the final medium) in such amount as to reach 625 μ M in the final medium. The cholesterol was dried under N₂ while swirling the vial to achieve maximal dispersion on the glass surface in contact with the medium. The 5mM m β -CD tissue culture medium was added to the dried cholesterol, vortexed to help resuspension, sonicated for 3 minutes in a 37C waterbath, and rotated overnight in a 37C waterbath under constant low flow of N₂. The pH was then adjusted to 7.4, and the solution was filter-sterilized through a 0.2 μ m filter (with prefilter). Note that even though the concentration of cholesterol used for making the complexes is 625 μ M, in practice, the final cholesterol concentration is unknown because the solution is saturated in cholesterol and the excess is filtered out.

2.2.6 Rat hepatic lipase (rHL) ELISA

The level of rHL enzyme in sonicated cell lysate or in medium was determined by ELISA using the method described by Cisar and Bensadoun (156) with the following

specifications: the plate was incubated overnight at 4°C after coating (1 µg/well of goat anti-rHL) to decrease background, and after sample addition to increase the signal due to specific binding. After addition of the blocking solution and of the conjugate, the plate was incubated for 2 hours at 37°C since longer incubation times were found to increase the background noise. The plate was washed 3 times after each step except after the conjugate step where 6 washes were found to be more optimal. Note that this assay does not cross-react with human HL, human LPL, or chicken LPL. The sensitivity of the rHL ELISA (0.05 ng) enables quantitative analysis of the secreted rHL for comparison with controls even for short-term treatments. Most secretion levels are normalized to cellular DNA concentrations and expressed as ng secreted rHL/mg cell DNA.

2.2.7 Determination of cell DNA concentration

Upon collection, cells were rinsed with PBS, the PBS was carefully aspirated, and the cells were scraped and collected in 750-1000 µL per 35 mm dish of 4 mM 3-(3-cholamidopropyl)diethyl-ammonio-1 propanesulfonate (CHAPS) lysis buffer (4 mM CHAPS, 50 mM NH₄OH, 3 U/mL heparin, pH 8.1, with freshly added 1 µg/mL leupeptin, 1 µg/mL antipain, 10 µg/mL benzamidine, 10 KIU/mL trasylol from 1000X stock in trasylol solvent, and 1 µg/mL chymostatin, 1 µg/mL pepstatin from 1000X stock in DMSO). The cell lysates were frozen at -20°C until analysis. Before the DNA assay, cell lysates were sonicated twice at 100 W for either 20 or 30 seconds each time, with a minimum of 30 seconds on ice in between. DNA concentration was assayed using a protocol adapted from Labarca and Paigen (157).

2.2.8 Extraction of free and total cholesterol from cell lysates for gas chromatography

Cell lysates (600 µL in 4 mM CHAPS lysis buffer as previously described) were sonicated twice for 20 seconds each with a ≥30 second interval on ice in between and transferred to acid-washed glass screw-cap tubes. Each lysate was extracted with 10 mL (1 volume) chloroform: methanol 2:1 along with 25 µg β-sitosterol (a soybean sterol

absent in mammals) as an internal standard. After 30 minutes on ice, 0.2 volume of water was added, and the mixture was vortexed and spun at 1000rpm for 5 minutes (at room temperature (RT) or at 4°C).

Three quarters of the ~6mL lower chloroform phase were dried and resuspended in 50μL chloroform for analysis of free cholesterol (FC).

A 1.5mL aliquot of lower phase was transferred to a new acid-washed screw-capped flat-bottom glass tube, dried under nitrogen in a ~40-60°C sand bath, saponified by incubating for one hour at 60°C in 2mL (1 volume) of 2% KOH in 95% ethanol, allowed to cool to room temperature, extracted with 1 volume hexanes and 1 volume water, and centrifuged for 5 minutes at 1000rpm at RT. The upper hexane phase containing the saponified lipids was transferred to new tubes, dried under nitrogen and resuspended in 50μL chloroform for analysis of total cholesterol (TC).

2.2.9 Gas chromatography

2μL aliquots of either FC or TC were analyzed by gas chromatography (158) using a hydrophobic RTX5 capillary column crossbonded SE-54 (Restek #10221, Lot# 1649A, 15m x 0.32mm inner diameter), and H₂ as a carrier gas. The column was set at 220°C in a 5890A gas chromatograph (Hewlett Packard) equipped with a flame ionization detector. The injector port and detector were maintained at 260°C and the hydrogen flow rate through the column was 34.5mL/min.

To determine the initial amounts of cell cholesterol, the R_f (response factor) value for β-sitosterol (S), the internal standard, was set at 1, and the R_f value for cholesterol (C) was determined to be equal to 1.01. Knowing that $C_{\text{count}}/C_{\text{mass}} = S_{\text{count}}/S_{\text{mass}} \times R_f$, for each sample the amount of cell free or total cholesterol (μg FC or TC/μg DNA) was determined using the following calculation:

$$[(FC/TC_{\text{count}} \times 25\mu\text{g})/(R_f \times S_{\text{count}})]/\mu\text{gDNA}.$$

The DNA mass for this calculation was determined in 600μl lysate.

2.2.10 Hepatic lipase activity assay

HL activity was determined using a protocol adapted from Bensadoun et al. (159). Briefly, tritiated triolein (in chloroform supplemented with 50 μ g/mL butylated hydroxytoluene) was weighed along with cold triolein to the desired specific activity. The chloroform was evaporated under a flow of nitrogen, and the vial was placed on ice. Gum Arabic was then added to 0.5% final in the 0.5mL final reaction and the mixture was emulsified by sonicating 3 times for 30 seconds each at 100W, with 30 seconds on ice in between each pulse. NaCl, BSA, and Tris-base were added to final concentrations of 1M NaCl, 1% BSA, and 0.2M Tris, pH 8.6. The final volume was adjusted with water to 0.5mL/reaction taking into account the volume of sample (≤ 0.2 mL) to analyze. This mix was used on the same day for quantitative analysis, but could be stored at -20°C for several months for semi-quantitative results. Each aliquot was placed on ice up to several hours until the enzyme sample was added. The reaction was vortexed and incubated in a 30°C waterbath for 1 hour. The reaction was stopped by addition of 6.5 volumes of Belfrage solution (methanol 30.8% w/w, heptane 50% w/w, chloroform 18.7% w/w, oleic acid 0.1g/L) and 1.5 volume of borate buffer (0.25M potassium tetraborate, 0.25M potassium carbonate, pH 9.5). The tubes were vortexed and centrifuged (10 minutes, $\sim 800g$, RT). 1.9mL of upper aqueous phase containing the liberated ^3H -labeled FFA were counted in scintillation liquid.

Calculation of catalytic activity:

$$\underline{\mu\text{Eq FA} / \text{hour} / \text{mL sample}} = \text{net cpm} \times 2 / (\text{Volume} \times \text{Time} \times 0.38 \times \text{SSA} \times 1.9)$$

where: cpm represents the number of counts per minute,

Volume is the volume of the reaction mixture expressed in mL,

Time is the reaction time in hours,

SSA is the specific activity of the substrate in cpm/ μ Eq FA.

2.3 Results

2.3.1 Linearity of rHL secretion over 24 hours

First, an assay was designed in order to verify that rHL secretion from cultured cells was linear over 24 hours in the conditions used for secretion assays throughout this thesis. Cells were treated with complete or LPDS medium supplemented with heparin and the medium was collected after 2, 4, 12 or 24 hours for analysis of secreted rHL by ELISA. rHL secretion was found to be linear in both complete ($y = 1.56x - 2.40$, $R^2 = 0.995$) and LPDS ($y = 1.10x - 0.32$, $R^2 = 0.999$) medium in the presence of 100U/mL heparin from 2 to 24 hours (Figure 2-1).

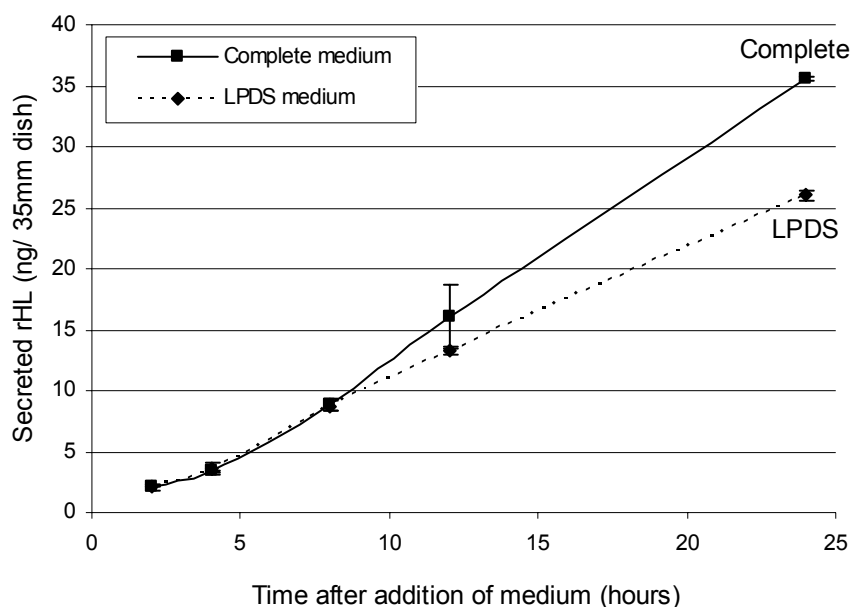


Figure 2-1 Linearity of rHL secretion over 24 hours in complete or LPDS media in the presence of 100U/mL heparin.

Fu5AH cells were split into 35 mm dishes. On the next day, at $t = -2$ hr, cells were placed on LPDS medium (10% LPDS, 2mM L-Glutamine in MEM) for 2 hours. At $t = 0$ hr, cells were rinsed with PBS containing 100U/mL heparin for 3 minutes, and this rinse was carefully aspirated before 2mL/well fresh complete or LPDS medium was added, each supplemented with 100U/mL heparin. Medium was collected at the indicated times, and analyzed for secreted rHL mass by ELISA.

2.3.2 Dual effects of depletion of cell cholesterol on rHL secretion

Depletion of intracellular cholesterol is reported to be a relatively slow process, taking about 12 hours. Thus, rHL secretion from cultured hepatoma cells was evaluated over the course of 24 hours, using increasing amounts of methyl- β -cyclodextrins, which can efficiently stimulate efflux of cholesterol from the cells (Figure 2-2). rHL secretion was found to increase to 173% of basal level over the first 4 hours on defatted medium. Over the next 4 hours, rHL secretion was further increased to 185 or 193% of basal level in defatted medium with no or small amounts (0.5mM) of methyl- β -cyclodextrins respectively, and this increased secretion level was maintained over the next 20 hours. On the other hand, in defatted medium containing 1mM or more of methyl β cyclodextrins, rHL secretion was found to be decreased compared to defatted medium only, down to 90.9% of basal level on 5mM for 20 hours. Microscopic observation of the cells under those conditions showed gross changes in cell morphology (data not shown), presumably due to a depletion of membrane cholesterol. Overall, the first effect of cholesterol depletion was to increase rHL secretion. On the other hand, over periods of depletion longer than 4-8 hours, the more stringent the cholesterol depletion, the faster rHL secretion levels decreased back to basal levels.

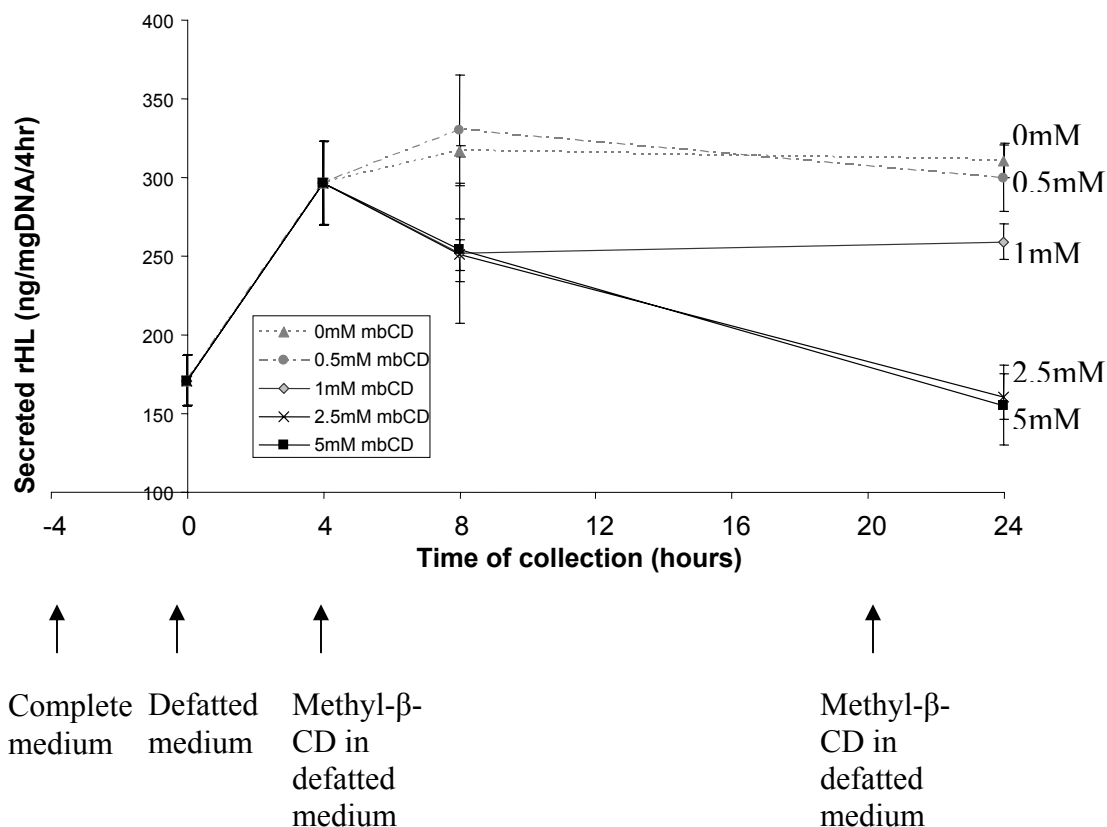


Figure 2-2 rHL secretion upon cholesterol depletion

McA-RH7777 cells were split into collagen-coated 35mm dishes. Upon confluency, at $t=-4$ hr, cells were placed on 2mL/dish fresh complete McA medium. At $t=0$ hr, the medium was aspirated and the cells were placed on 2mL/dish defatted medium (5% defatted FBS, 2mM L-glutamine in DMEM). At $t=4$ hr, the defatted medium was aspirated and the cells were placed on fresh defatted medium supplemented with increasing amounts of methyl- β -cyclodextrin as indicated. Sets of 3 dishes were collected at times 0hr, 4hr, 8hr and 24hr. In each case, four hours prior to collection, the cells were rinsed with 100U/mL heparin-containing PBS for 3 minutes, this wash was carefully aspirated, and the cells were incubated for 4 hours with 1mL/dish of the indicated treatment medium supplemented with 100U/mL heparin. After the four-hour incubation, the medium was collected and spun at 14,000rpm for 10 minutes at 4C. The supernatant was transferred to new tubes and frozen at -20C before being analyzed by ELISA. The cells were rinsed with PBS and collected in 0.75mL of 4mM CHAPS lysis buffer for storage at -20C. The cells were thawed and sonicated before the DNA was analyzed. rHL secretion levels are presented as ng medium rHL/mg DNA over the four hour secretion period.

2.3.3 Decreased rHL secretion upon repletion of cell cholesterol

Based on the previous result, in order to ensure a high level of basal rHL secretion, cells were incubated for 4 hours in defatted medium supplemented with 2mM methyl- β -CD. After this preliminary cholesterol depletion phase, rHL secretion levels were monitored upon repletion with cholesterol (5mM methyl- β -CD 8:1 cholesterol) in 25% defatted medium (Figure 2-3). Cholesterol repletion led to a rapid decrease in secreted rHL down to 56.6% and 17.5% of time 0 by 4 and 48 hours respectively.

In the same experiment, free and total cholesterol levels were assessed by gas chromatography (Figure 2-4). As expected, total and free cholesterol levels were low at time 0 (after cells had been depleted of cholesterol for 4 hours), and the methyl β cyclodextrin:cholesterol complexes increased cell cholesterol levels throughout the course of the experiment. Free cholesterol levels increased by 5.6 fold by 4 hours and remained at that high level thereafter. Presumably, free cholesterol levels are maintained within a narrow range of concentrations within the cells, and all excess cholesterol is stored in the cells as cholesterol esters. Accordingly, total cholesterol increased by 7.0 fold by 4 hours and kept increasing over the course of the experiment up to 25.9 fold of original levels at 24 hours.

In order to confirm these results and verify that the downregulation of secreted rHL was not merely due to prolonged exposure to defatted serum-containing medium, parallel experiments were carried out with cells in medium containing either 5% defatted FBS, or 25% lipoprotein-deficient serum (LPDS, 20% from horse serum and 5% from FBS). During the cholesterol repletion phase, rHL secretion dropped down to 17.5% (Figure 2-4) or 16.1% (Figure 2-5) of basal levels with 5mM methyl- β -cyclodextrins in 25% or 5% defatted medium respectively. However, 5% defatted serum medium or 25% LPDS medium alone (without cholesterol addition) were also found to decrease rHL secretion following the 4 hour depletion phase, albeit to a lesser degree, to 50.9% and 63.0% of basal levels respectively. This result was not unexpected given the dual effects of

cholesterol depletion on rHL secretion (Figure 2-2). The fact that the LPDS medium had a very similar effect to the defatted medium suggests that the observed effect is not just due to the mode of preparation of either defatted serum (by Cab-O-Sil treatment and n-butanol/isopropyl ether extractions) or lipoprotein deficient serum (by potassium bromide ultracentrifugation). Overall, cholesterol significantly decreases rHL secretion to 36.8% and 31.7% of control 5% defatted medium at 24 and 48 hours respectively. Thus, cholesterol repletion decreased rHL secretion, at least at 24 and 48 hours within the repletion phase. Whether the decrease at 4 hours within the repletion phase is due to cholesterol or to the secondary effects of the original cholesterol depletion is not clear.

In order to further confirm that the observed effects are indeed due to the manipulation of sterol content within the cells, cholesterol was presented to the cells under different forms. Whether presented in the form of lipoproteins (in complete medium), a mix of sterols (10 μ g/mL cholesterol and 1 μ g/mL 25-hydroxy-cholesterol from an alcohol concentrated stock) or as a complex with methyl- β -cyclodextrins, cholesterol was found to decrease rHL secretion slightly after 24 hours compared with control LPDS medium (data not shown). Similar results of the sterol mix were observed in an enriched basal medium (OPTI-MEM), but because secretion levels were overall lower than in the traditional MEM basal medium, this medium was not used in further experiments.

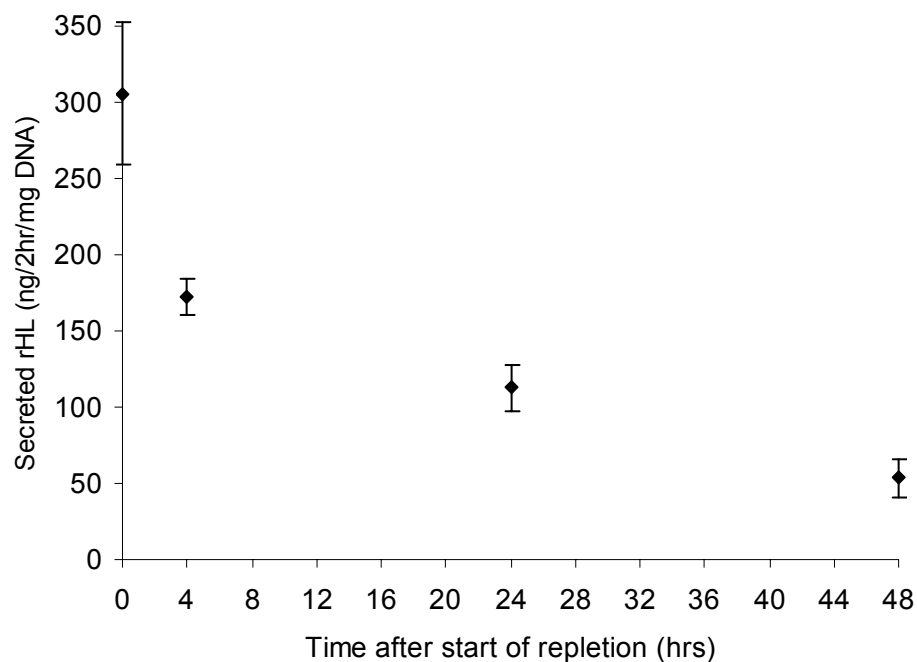


Figure 2-3 rHL secretion upon cholesterol repletion

McA-RH7777 cells were split into collagen-coated 35mm dishes. At $t=-4$ hr, to deplete the cells of cholesterol, the cells were placed on 2mM methyl- β -CD in defatted medium. This medium was refreshed at $t=-2$ hr. At $t=0$ hr, the medium was aspirated and replaced by 5mM methyl- β -CD 8:1 cholesterol (molar ratio) in 25% defatted medium. This medium was refreshed at $t=24$ hr. For each secretion time point, the medium was aspirated on 3 dishes, and the dishes were rinsed with PBS containing 100U/mL heparin, then 0.75mL of the respective treatment medium containing 100U/mL heparin was added. Medium and cells from these dishes were collected 2 hours later at the indicated time point. The medium was analyzed for rHL mass by ELISA, and the cell lysates were analyzed for DNA. Averages \pm SD, $n=3$.

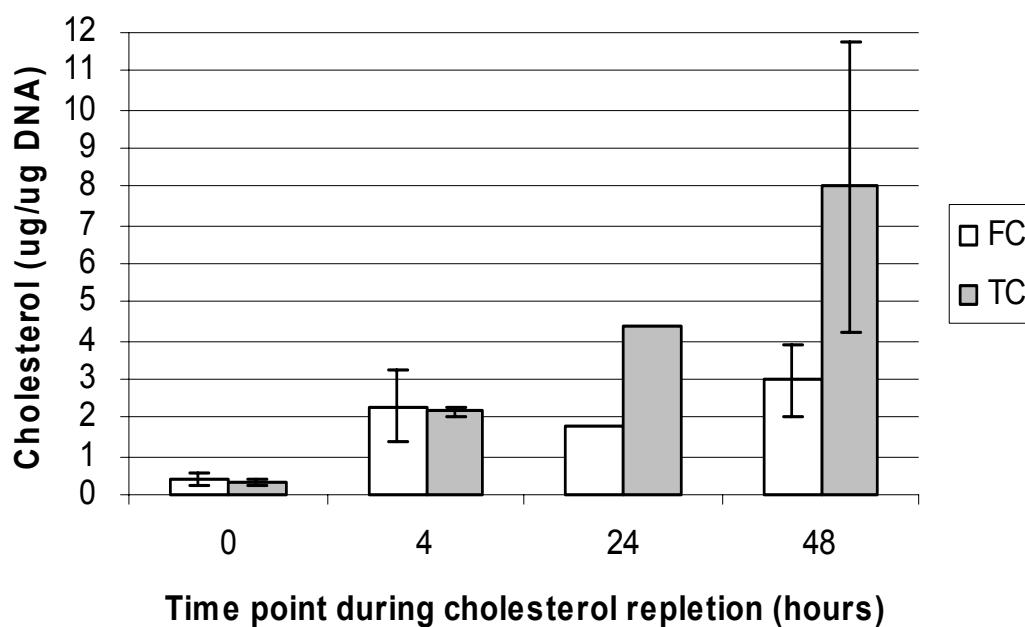


Figure 2-4 Total and free cholesterol levels upon cholesterol repletion

Cell lysates from McA-RH7777 cells (as described in Figure 2-3) were sonicated and extracted with chloroform:methanol 2:1 along with β -sitosterol as an internal standard. Most of the lower phase was dried and resuspended in chloroform for analysis of free cholesterol. The remaining lower phase was dried under nitrogen, saponified, and extracted with hexanes and water. The upper hexane phase was dried under nitrogen and resuspended in chloroform for analysis of total cholesterol. Both free (FC) and total (TC) cholesterol analyses were done by gas chromatography. Average \pm SD, n=3.

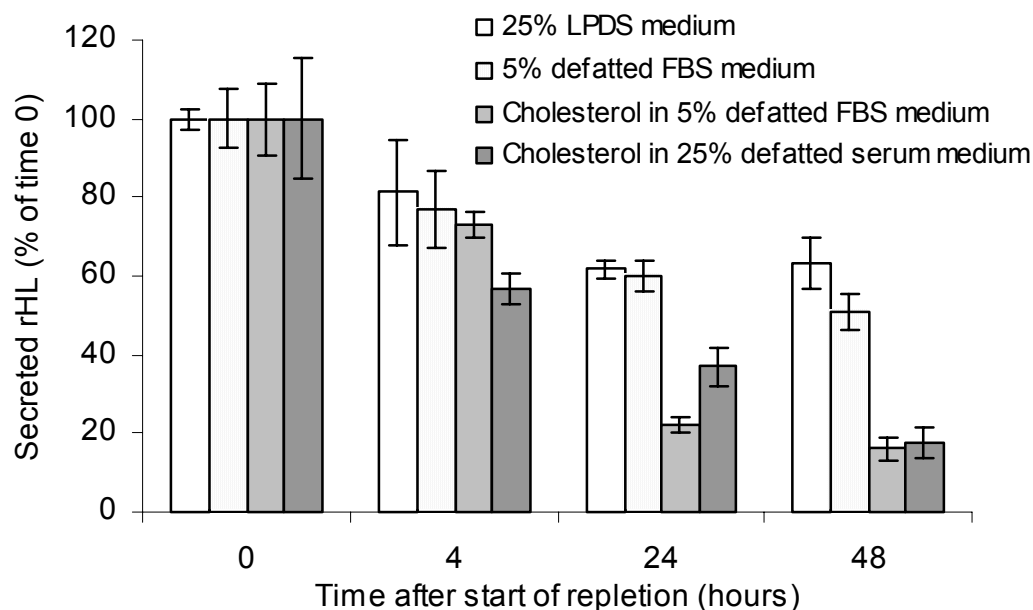


Figure 2-5 rHL secretion upon cholesterol repletion or upon prolonged cholesterol depletion

At $t=-4$ hr, McA-RH7777 cells in 35mm dishes were placed on 2mM methyl- β -CD either in delipoproteinated or defatted serum medium (as in subsequent treatment) in order to deplete the cells of cholesterol. This medium was refreshed at $t=-2$ hr only in the experiment with cholesterol in 25% defatted serum medium. At $t=0$ hr, the medium was aspirated and replaced by either 25% LPDS serum (20% from horse serum and 5% from FBS) medium, 5% FBS defatted serum medium, 5% FBS defatted medium supplemented with 5mM methyl- β -CD 8:1 cholesterol (molar ratio), or 25% defatted serum (20% from horse serum and 5% from FBS) supplemented with 5mM methyl- β -CD 8:1 cholesterol. All treatment media were refreshed at $t=24$ hr. For each secretion time point, the medium was aspirated on 3 dishes, the dishes were rinsed with PBS containing 100U/mL heparin, and 0.75mL of the respective treatment medium containing 100U/mL heparin was added. Medium and cells from these dishes were collected at the indicated time point after a two-hour incubation. The medium was analyzed for rHL mass by ELISA, and secretion was normalized for DNA. Each bar corresponds to the average secreted rHL mass as a percent of secretion at time 0 for each series, \pm SD ($n=3$).

This graph is a compilation of data from different experiments, where the experiment with cholesterol in 25% defatted serum medium is the same as the one presented in Figure 2-3 in absolute secretion values.

2.3.4 Increases in intracellular free cholesterol can mimic the effects of cholesterol treatment

Free cholesterol concentration is more critical than total cholesterol concentration to exert sterol-mediated regulatory effects. Thus, we sought to determine whether an increase in intracellular free cholesterol could mimic and/or amplify the effects of cholesterol treatment. The Acyl Coenzyme A: Cholesterol Acyltransferase (ACAT) inhibitor 58-035 at concentrations $>0.1\mu\text{g/mL}$ in combination with cholesterol-loading agents is known to selectively and effectively inhibit the esterification of cholesterol ($>95\%$ within 30 minutes in Fu5AH cells), leading to a slight increase in cellular FC, but overall decreasing total cell cholesterol (115).

As expected, increasing concentrations of 58-035 for 24 hours decreased rHL secretion (Figure 2-6). Because high amounts of 58-035 can lead to cell apoptosis over long incubation times, the smallest amounts of 58-035 to achieve full effect on rHL secretion (2 or $5\mu\text{g/mL}$) were used in later experiments.

Next, cells were treated either with sterols, 58-035, or a mixture of both to see whether the combination of sterols and an inhibitor of cholesterol esterification could amplify the decrease in rHL secretion. There appeared to be no additive effect of sterols and 58-035 on rHL secretion (Figure 2-7) suggesting that they may act at least partly through the same pathway, and that “full” inhibition of rHL secretion through this pathway is achieved under all treatment conditions.

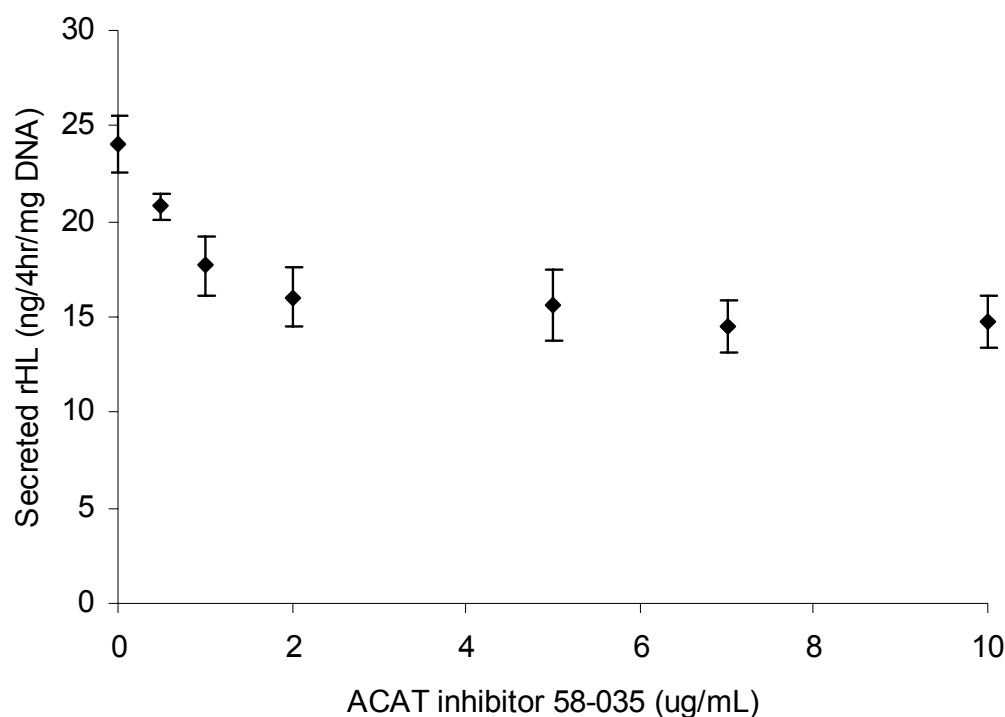


Figure 2-6 ACAT inhibitor 58-035 decreases rHL secretion in a dose-dependent fashion

Fu5AH cells were spit into 35mm dishes. Upon confluency, at t=0hr, the medium was aspirated and the cells were placed on 2mL/well treatment medium, which consisted of Fu5AH LPDS medium (10% LPDS, 2mM L-Glutamine in MEM) supplemented with the indicated concentration of ACAT inhibitor or 0.1% DMSO as a carrier. At t=20hours, the medium was carefully aspirated and replaced by 1mL/well of an identical treatment medium supplemented with 100U/mL heparin. After a 4 hour incubation, at t=24hours, the medium was collected and centrifuged for 10 minutes at 15,000rpm, then the supernatant was transferred and frozen at -20C until the rHL ELISA was performed. The cells were rinsed with PBS, scraped in 1mL/well of 4mM CHAPS lysis buffer, frozen at -20C, and sonicated twice for 20-seconds at 100W before DNA was analyzed for normalization. Average \pm SD, n=3.

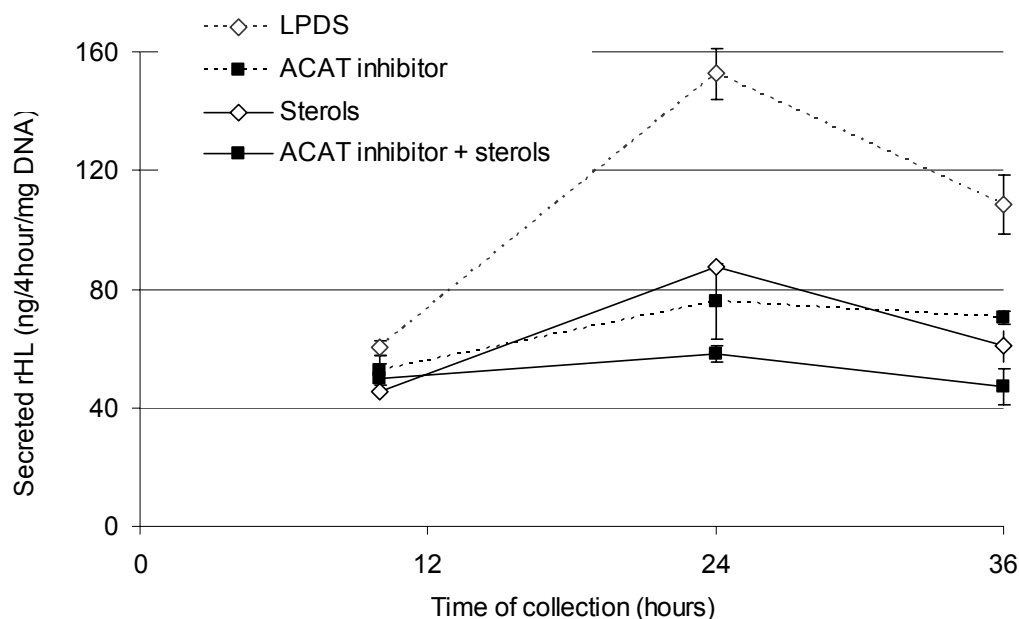


Figure 2-7 The decrease in rHL secretion upon 58-035 and sterol treatment is not additive

Fu5AH cells were split into 35 mm dishes. Upon confluency, at t=0hr, cells were placed on 1.5mL/dish LPDS medium (10% LPDS, 2mM L-Glutamine in MEM) alone (LPDS), or LPDS medium supplemented with 5 μ g/mL 58-035 (ACAT inhibitor), with 10 μ g/mL cholesterol and 1 μ g/mL 25-OH-cholesterol (Sterols), or with a combination of both (ACAT inhibitor + sterols). Ethanol or DMSO were added as carrier controls as appropriate. Four hours prior to each collection, the medium was aspirated from the cells to be collected and replaced by 1mL/dish fresh treatment medium supplemented with 100U/mL heparin. Medium and cells were collected at the indicated times. The medium was analyzed for secreted rHL mass by ELISA, and the cells were analyzed for DNA content. Average \pm SD, n=3.

2.3.5 Cholesterol does not affect cell HL specific activity

Hepatic lipase is an enzyme which catalytic activity can potentially be regulated; therefore, we sought to determine whether sterol regulation of HL also involved regulation at the activity level. Cell-associated rHL specific activity was not significantly decreased by cholesterol administered in the form of 5mM methyl- β -cyclodextrins complexed at an 8:1 molar ratio cyclodextrin:cholesterol for 17 hours (Figure 2-8). Further, cell-associated rHL specific activity was not significantly changed by sterols

addition, 58-035 treatment, or a combination of both 58-035 and sterols (Figure 2-9), while the ACAT inhibitor did decrease secreted rHL mass down to 52% in this experiment (data not shown). Overall, cell sterol content manipulation was found to have no significant effect on cell-associated HL specific activity.

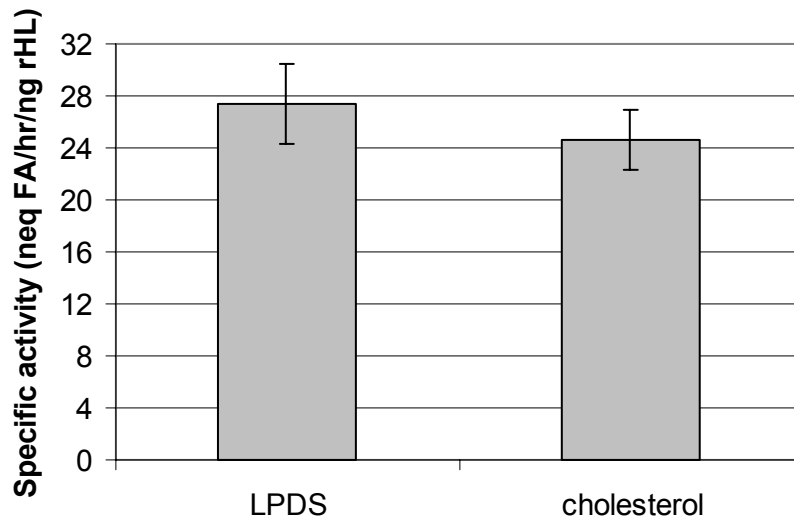


Figure 2-8 Cholesterol does not affect the specific activity of intracellular and cell-associated hepatic lipase

McA-RH7777 cells were split into collagen-coated 100mm dishes and placed on Cab-O-Sil-treated LPDS medium at confluency. After 4 hours on LPDS medium, at t=0hr, cells were placed on LPDS medium with or without supplementation with 5mM methyl- β -cyclodextrin complexed at an 8:1 molar ratio cyclodextrin:cholesterol. At t=17hr, the cells were collected on ice and sonicated before being immediately analyzed for lipase activity. The remainder of the samples was frozen at -20C and later analyzed for rHL mass (by ELISA). The bars correspond to the average specific activity of triplicates \pm standard deviation. $p=0.28$, two-tailed unpaired t-test.

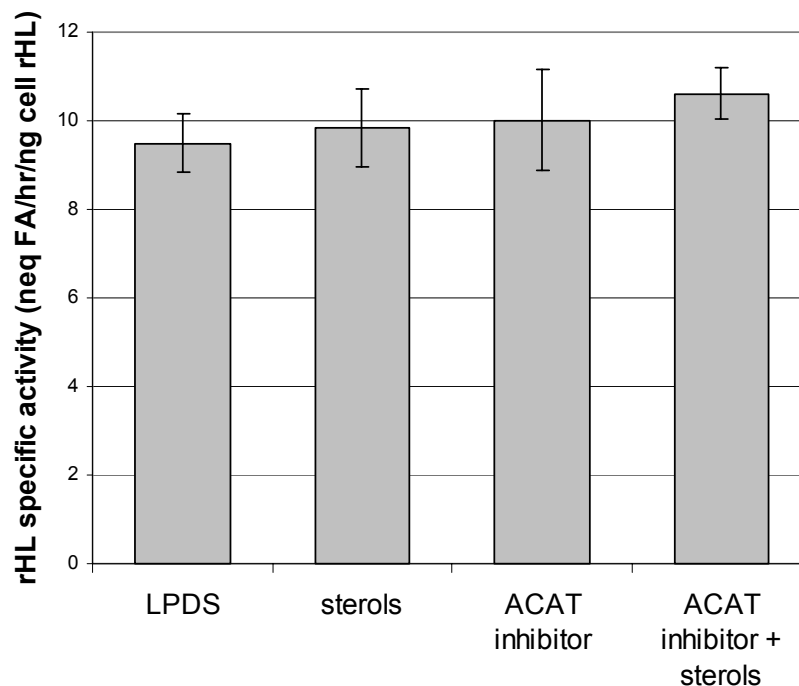


Figure 2-9 Intracellular and cell-associated hepatic lipase specific activity is not affected by sterol or 58-035 treatments

Fu5AH cells were split into 100mm dishes. Upon confluency, at t=0hr, the medium was aspirated and the cells were placed on 7mL/dish LPDS medium (MEM, 2mM L-glutamine, 10% LPDS) supplemented with either 2µg/mL ACAT inhibitor (using DMSO as a carrier) or 10µg/mL cholesterol +1µg/mL 25-OH-cholesterol (using ethanol as a carrier at 1:500 dilution in cell media) or both. Carrier controls were added as appropriate. At t=24hours, the medium was collected and pooled per sets of 3 dishes, then frozen at -20C before being assayed for secreted rHL mass by ELISA. The cells were rinsed with PBS and each set of 3 dishes was collected into 1mL of 4mM CHAPS lysis buffer. The lysate pools were sonicated twice for 30 seconds at 100W, and then spun down for 10 minutes at 14,000 rpm at 4C. The supernatants were transferred and immediately assayed for lipase activity. The remaining lysates were frozen at -20C before being assayed for rHL mass by ELISA. The data is expressed as nanoequivalent (neq) fatty acids released/hour/ng cell (intracellular and cell-associated) rHL. No significant difference was found ($p>0.09$) using Student's two-tailed unpaired t test.

2.4 Discussion

Previous reports from the literature have shown that cholesterol decreases HL levels in human hepatoma HepG2 cells (74). After confirming this result in HepG2 cells (data not shown), we turned to rat hepatoma cells for further studies. This switch was motivated by a practical reason : the ELISA for rat HL is a much more rapid, and more

importantly much more sensitive assay than the currently available “biotinylated” human ELISA. Nonetheless, cholesterol was expected to lead to a similar decrease in HL synthesis in rat cells than in human cells since cholesterol feeding in rats has consistently been found to lead to decreased HL synthesis in the literature (70-72).

In order to study the down-regulation of hepatic lipase by cholesterol, we first sought to find baseline conditions where the enzyme would be secreted at high levels. Hepatic lipase (activity and sometimes mRNA) has been reported to be upregulated in some conditions by insulin (160), triiodothyronine/T3 (161;162) and glucose (163), but these had no effect on HL secretion levels in our system (data not shown). Therefore, to find optimal conditions to study the effect of a cholesterol loading, we investigated the effect of a preliminary depletion of cellular cholesterol with LPDS, which also had previously been reported to increase HL activity secretion after 24 (74) or 48 hours (75) by up to 50%. Unexpectedly, in our model, a long-term (24-hour) incubation in LPDS medium as compared with complete medium decreased HL secretion (Figure 2-1). Of note, in this experiment, the medium was not changed over the 24-hour incubation, raising the possibility that unknown elements of the LPDS medium could have become limiting, therefore decreasing HL secretion independently of cholesterol levels. In contrast, as shown in Figure 2-2, we found that depleting cells of cholesterol in our model did enhance HL secretion under slightly different conditions. This time, in order to minimize the potential for factors other than cholesterol to become limiting in LPDS medium and affect secretion levels; secretion was measured only over the last four hours after addition of fresh treatment medium. When depletion was achieved in relatively mild conditions (i.e. defatted medium alone or supplemented with only 0.5mM methyl- β -cyclodextrins), cholesterol depletion yielded an increase in secreted HL within the first 4 hours of treatment, and these high levels of HL secretion were maintained over the course of the 24-hour treatment. However, when more stringent conditions were used for cholesterol depletion (i.e. defatted medium containing 1mM or more of methyl- β -cyclodextrins) rHL

secretion was found to be decreased again (compared to defatted medium alone) starting after 8 hours. As mentioned before, microscopic observation of the cells under those conditions showed gross changes in cell morphology. Because of this observation, it is likely that in these extreme conditions, membrane cholesterol is depleted. Indeed, stable levels of cholesterol within the cells are known to be essential for membrane integrity. In tissue culture, where rapidly dividing cells require active membrane synthesis, the cellular demand in cholesterol is high. Yet, instead of cholesterol flowing into the cells, at high levels of methyl- β -cyclodextrins cholesterol would be expected to shuttle from the cells to the medium, which could lead to severe consequences for cell membrane integrity. Alternatively, we cannot rule out that the delayed decrease in HL secretion under conditions of severe cholesterol deprivation could be due, at least partly, to the activation of SREBP, which in turn would lead to a decrease in HL synthesis (see chapter 3). Overall, depletion of cell cholesterol in hepatoma cells increased HL secretion in conditions where membrane integrity was maintained. A similar mild technique for cholesterol depletion was frequently used in subsequent experiments in order to increase HL baseline secretion levels.

Whether using rat hepatoma cells (Fu5AH or McA-RH7777 cell lines) or human hepatoma cells (HepG2 cells – data not shown) as a model, cholesterol repletion was uniformly found to decrease HL secretion, and this effect was consistently seen early on, starting at 4 hours after addition of cholesterol. Furthermore, intracellular cholesterol was modulated using different means: by culturing cells in the presence of lipoprotein-containing medium versus delipoproteinated medium; by adding cholesterol directly to the medium as a complex to methyl- β -cyclodextrin or as a mixture of 10 μ g/mL cholesterol and 1 μ g/mL 25-OH-cholesterol (a mixture commonly used for the study of sterol-regulated genes such as the LDL receptor or HMG-CoA-reductase); by adding a mixture of sterols and the ACAT inhibitor 58-035 simultaneously to the medium (which increases cell free cholesterol levels while slightly decreasing total cholesterol levels

(115); or finally by adding the ACAT inhibitor alone. Either of these means of increasing cellular total or free cholesterol levels led to a decrease in secreted HL. Further, the fact that HL secretion levels decreased in response to an ACAT inhibitor strongly suggested that it is an increase in intracellular free cholesterol specifically, and not an increase in total cholesterol, which mediates the decrease in HL secretion.

Overall, the results presented here are consistent with previous results in the literature. After a 48 hour pretreatment in LPDS medium, treatment with 1mM mevalonic acid (an intermediate in the cholesterol biosynthesis pathway) for 48 hours increased cell cholesterol by 26% and resulted in a 53% decrease in secreted HL activity (compared with LPDS medium control) (74). However, in contrast to our study where a mixture of cholesterol and 25-OH-cholesterol decreased HL secretion, after a 48 hour pretreatment in LPDS medium, exposure of cells to 25-hydroxycholesterol (an oxidized metabolite of cholesterol) for 24 hours produced a small but reproducible induction of secreted lipolytic activity (74). The reasons for these discrepancies are unknown.

We sought to determine whether cholesterol also had an effect on HL specific activity. While decreasing secreted HL mass, we found that cholesterol did not affect cell-associated and intracellular HL specific activity. The reason why the activity assay was done on cell lysates as opposed to medium is due to the lower sensitivity of the lipase activity assay compared to that of the ELISA. The concentration of HL in medium incubated with cells for a period of 4 hours did not allow for a reliable quantification of medium enzyme activity. On the other hand, the instability of HL enzyme activity at 37C precluded the use of longer incubation times in order to allow for HL medium concentration to increase sufficiently.

The roles of hepatic lipase in lipoprotein metabolism stem from both its enzymatic role as a phospholipase and a triglyceride hydrolase and from its “bridging” role to increase the residency time of lipoproteins in close proximity to their cellular receptors (23). Its combined role as a lipolytic enzyme and as a ligand increases both the hepatic

clearance of chylomicron remnants and other apo-B containing lipoproteins (23;25;26;164;165), as well as the selective uptake of HDL-CE in the liver (28;166-170). As a consequence, high levels of extra-cellular HL have been shown to increase cholesterol internalization from circulating lipoproteins in liver and steroidogenic cells. The effects of cholesterol depletion or repletion on HL levels are amplified by its catalytic role in lipid metabolism, such as a small decrease in secreted HL could lead to a substantial decrease in its propensity to increase cellular internalization of cholesterol. This hypothesis suggests that hepatic lipase could play a role in maintaining cellular cholesterol homeostasis. Under conditions of low free intracellular cholesterol levels, HL secretion levels would increase, which would favor cholesterol influx from the circulation into the cells. However, when intracellular free cholesterol levels are high, HL secretion would be down-regulated, which would limit the amount of cellular cholesterol uptake. Overall, the down-regulation of HL by cholesterol would be beneficial in the liver, and potentially in other cell types where HL has been shown to be expressed, albeit to a lesser extent, such as the adrenals, ovaries and macrophages. If these results could be extrapolated to macrophages, this would have special significance in the context of the atherosclerotic plaque. As opposed to HL, LPL is activated by high levels of cholesterol through activation of LXR (171), and macrophage expression of LPL has been shown to contribute to foam cell formation and atherosclerosis *in vivo* (172). In the case of HL, the down-regulation of HL by sterols would tend to attenuate this feed-forward lipid-loading mechanism.

In the next two chapters, we sought to further investigate the mechanisms by which cholesterol down-regulates hepatic lipase secretion. In Chapter 3, we examined the effect of cholesterol on HL transcriptional levels, and in Chapter 4, we examined the effect of cholesterol on HL at the post-transcriptional level.

Chapter 3 - SREBP as a major mediator of HL transcription

3.1 Introduction

In the previous chapter, we defined the effect of cholesterol on HL protein and activity levels. In this chapter, we examined the effects of sterols on HL at the transcriptional level. We provide evidence that cholesterol decreases HL mRNA steady-state levels without affecting the mRNA degradation rate. Regulation of HL promoter activity was investigated through luciferase activity assays. Cholesterol as such failed to alter the activity of a proximal hHL promoter construct.

Within liver cells, intracellular cholesterol homeostasis is tightly maintained. Sterol Regulatory Element Binding Proteins (SREBPs) are the major transcription factors mediating the feedback regulation of cholesterol levels by controlling the expression of genes involved in cholesterol biosynthesis (e.g. HMG-CoA reductase), lipoprotein uptake (LDL Receptor), or (62) lipoprotein hydrolysis and uptake (lipoprotein lipase (LPL)). HL is known to enhance lipoprotein uptake and cholesterol loading in cell culture (23;25;28). Therefore, it became apparent that SREBPs may also play a significant role in the cholesterol regulation of HL expression.

Evidence is presented in this chapter that nuclear SREBPs (nSREBPs) inhibit the transcription of the HL gene and could be mediating the downregulation of HL under conditions where cholesterol is elevated and/or cholesterol synthesis is inhibited. Cholesterol feeding in mice results in decreased concentrations of nSREBP2 and nSREBP1a but in a marked increase in nSREBP1c (126). What is more, nSREBP transfection could reverse the activation of HL by USF1 in promoter activity assays. Several activator binding sites within the hHL proximal promoter were identified through gel shift assays, but these assays failed to show direct binding of nSREBP to the HL

promoter. Co-immunoprecipitation experiments suggest that inhibition of transcription may occur through interaction of SREBP1 with positive effectors of HL transcription such as USF1.

3.2 Methods

3.2.1 Reagents

Please refer to the previous chapter for commonly used reagents. Actinomycin D (Sigma A9415) was prepared as a 1.25mg/mL (2,500X) stock in ethanol and stored at 4°C. The final contribution of ethanol due to actinomycin D in the treatment medium was 0.04%. N-acetyl-leucyl-leucyl-norleucinal (ALLN) was obtained from Sigma (A-6185) and was stored at –20°C as a 10mg/mL stock in DMSO. Mevastatin/compactin (Sigma M2537) or lovastatin (Fisher NC9702522) were made as a 500 or 1000X stock in ethanol and were stored at –20°C.

3.2.2 Plasmids

The pCMV-SREBP1a-460 vector was purchased from the ATCC (#99637). pCMV-SREBP1c-436 was obtained from Dr. M-J Latasa in Dr. Sul's laboratory (U.C. Berkeley) and could be obtained from the ATCC (#99636). Both vectors originated from the laboratory of Drs. Joseph Goldstein and Michael Brown (University of Texas Southwestern, Dallas, TX) (120).

pSRE-luc (pSynSRE) was received from Tilla Worgall, and originated from Tim Osborne's group (173). This construct contains a pGL2-basic backbone with a TATA box and transcription start from a SalII-HindIII fragment (-28/+39) of Synthase mut P inserted into XhoI/HindIII of the multiple cloning site. Just upstream is a -324/-225 fragment of hamster HMG-CoA synthase in between SacI and NheI, which contains two sterol responsive elements (SREs) required for sterol regulation, binding sites for the ATF/AP1 family of transcription factor, and a consensus binding site for CBF/NFY. Thus this vector is specifically activated by SREBPs and it is suited for monitoring the transcriptional activity of the mature nuclear form of SREBP (nSREBP).

pcDNA3-USF1 and pcDNA3-USF2, mammalian expression vectors for human USF1 and mouse USF2 respectively, were obtained from Dr Sul's laboratory (U.C. Berkeley) and originated from Dr. M. Sawadogo (M.D. Anderson Medical Center, University of Texas Southwestern, Dallas). The pFlag-dominant negative USF1 construct was a gift from Dr. Sul's laboratory (U.C. Berkeley). This vector is a pcDNA3.0 construct driving the expression of an N-terminus flagged human USF1 with an internal deletion of the basic region of USF1 from amino acid 193 to 211 (174). Without this DNA binding sequence, the mutated USF1 can dimerize but cannot bind DNA and activate transcription.

pRc/CMV and pcDNA3 were purchased from Invitrogen.

pEGFP-N1, a mammalian expression vector for the green fluorescent protein, was obtained from Clontech (#6085-1).

3.2.3 Tissue culture

Fu5AH and McA-RH7777 cells were handled as described in the previous chapter. Human hepatoma HepG2 cells were obtained from the Children's Memorial hospital in Boston and were grown in the same medium as Fu5AH medium (10% FBS, 2mM L-glutamine, 11.7g/L NaHCO₃, 12.7g/L HEPES, 50.9g/L GIBCO #61100-103 MEM, pH 7.2). A 1:2 splitting ratio was generally used for confluency to be reached again within 2-3 days.

3.2.4 Isolation of RNA

RNA was isolated with Trizol (Invitrogen) using a protocol adapted from the manufacturer's instructions. Using this protocol, the yield was generally ~100-300µg RNA/100mm dish or ~30-45µg RNA/35mm dish for confluent McA-RH7777 cells.

Briefly, cells grown in 35mm dishes were harvested in 1.25mL Trizol™. The lysates were transferred to ribonuclease (RNase)-free eppendorfs and incubated at RT for 5 minutes. Optionally, the lysates were stored at -80°C at this step. For phase separation, 0.25mL chloroform was added to each tube, the tubes were shaken vigorously for 15

seconds, incubated at RT for 3 minutes, and spun at 11,300rpm (12,000g) for 15 minutes at 4°C. The upper aqueous phase was transferred to clean RNase-free eppendorfs and the RNA was precipitated with 0.625mL/tube isopropyl alcohol. The samples were mixed by pipeting up and down, incubated for 10 minutes at RT, and centrifuged at 11,300rpm for 10 minutes at 4°C. The RNA-gel like pellet was washed with at least 1.25 mL/tube of 75% ethanol. The samples were spun at 9,000 rpm (7,500g) for 5 minutes at 4°C, the supernatants were removed and the samples were air-dried until the RNA became transparent. Each RNA sample was resuspended in 100µl RNase-free water.

Optionally, the RNA was further purified using RNeasy columns (QIAGEN). In this case, 100µg/sample (the maximum capacity of the columns) was purified according to the manufacturer's instructions, consistently with almost 100% recovery.

3.2.5 mRNA quantification by real-time reverse transcription – polymerase chain reaction (RT-PCR) using a TaqMan probe assay

Basic principle:

The TaqMan assays use a fluorogenic probe that anneals within the complementary DNA (cDNA) target sequence during polymerase chain reaction (PCR). Because the probe is designed to have a lower annealing temperature, it anneals prior to annealing of the primers. A reporter dye (FAM) is attached to the 5' end of the probe, and a quencher dye is attached to its 3' end. During PCR, the 5' nuclease activity of the DNA polymerase cleaves the probe, which releases the reporter dye from the quencher and increases the fluorescence intensity.

Reverse transcription reaction:

1µg RNA/sample was reverse transcribed in a 50µl reaction in the presence of 2.5µM oligo dT (AB # N808-0128) as primers, 1.25U/µl MultiScribe™ Reverse Transcriptase (Applied Biosystems #4311235, a recombinant Moloney murine leukemia virus (rMoMuLV) reverse transcriptase), 1X TaqMan RT buffer, 5.5mM MgCl₂, 500µM/dNTP (AB # N808-0260), and 0.4U/µl RNase inhibitor (Applied Biosystems). A no template

control (NTC) containing no RNA was always included as a negative control. The reactions were set up in 0.3mL thin-walled PCR tubes for the robocycler, mixed well, and spun down. Primers were annealed by incubating for 10 minutes at room temperature. The reverse transcriptase reaction was achieved by incubating at 48°C for 30 minutes, and the enzyme was then inactivated by a 5-minute incubation at 95°C. The obtained cDNA was frozen down at -20°C until real-time PCR was performed.

Real time polymerase chain reaction:

Real-time PCR using cDNA as a template was performed utilizing a custom-designed TaqMan probe assay (Assays-by-Design (SM) Service, Gene Expression, Assay ID 4331348) with two unlabeled PCR primers (RHL-E2E3F: CCA TCC ACT TGT CAT GAT CAT CCA and RHL-E2E3R: CTT CCA GAT CCA GGT TTC TAG CAA, 18μM each) and a FAMTM dye-labeled TaqMan® MGB probe annealing on the same strand as the reverse primer (FAM CAT CCA CCG ACC ACC C, 5μM). The probe was designed based on the corrected cDNA sequence from Sendak. The exon/intron junctions are not known for rat, but only those exon/exon junctions that bore 100% homology with the human sequence were sent as options for the design of a probe. The designed probe overlaps the putative junction of exon2 and exon3.

The assay and cycling conditions were set according to the manufacturer's instructions. Briefly, the 20μL final PCR reaction consisted of 1X TaqMan PCR Master Mix (AB #4324018 1), 1X assay mix (1:20 dilution of appropriate 20X probe and primers assay mix), and for the hepatic lipase detection samples 4μL/sample of either cDNA sample, standard, or NTC. When results were normalized to a negative control as opposed to being calculated from a standard curve, the ribosomal phosphoprotein P0 (36B4) (175) was used. Beta actin was found to respond to treatments and therefore could not be used as an internal control. For the 36B4 internal standard PCR reactions, the cDNA was first diluted 1:9 in water, and 9μL of this diluted cDNA was used in the 20μL reaction. The 384 well plate was covered with an optical adhesive cover and spun

down before the PCR. The ABI Prism 7900HT at the Biotechnology Resource Center facility was used for the runs. Real time thermal profile consisted of 2 steps, the first step being a one-time 10 minute incubation at 95C, and the second step consisting of 40x {(95C, 15 seconds), (60C, 1minute)}. ROX fluorescence was used as a passive reference for background.

Calculations for standard (when applicable):

The rHL cDNA used as a standard was inserted in pCDNA3.1(-) (Invitrogen). The number of rHL transcripts was calculated using the following formula:

$$\text{rHL transcript number} = \text{rHL standard mass (pg)} \times 10^{-12} \times (6.022\text{E}+23\text{copies/mole}) / \text{MW}$$
where the molecular weight of vector and insert is equal to 3641739.

Note that Applied Biosystems now has a hepatic lipase (LIPC) TaqMan Assay On Demand available (Rn00561474_m1).

3.2.6 Cloning of the human HL promoter constructs

Constructs containing deletions of the human HL (hHL) promoter (up to -1480nt) driving the expression of a firefly luciferase reporter gene were created by cloning into the pGL3 basic vector using a PCR-based approach. At the time when cloning was initiated, the available sequences were that of Chang et al. (Genbank X58779) (58) and Ameis et al. (M35425) (56) which overlapped and together covered 3446 base pairs (bp) of the hHL promoter upstream of the translation start codon. Note that the complete sequence of the (60-150kb) human hHL gene (promoter and introns) is now available from the chromosome 15 genomic contigs NT_010194 and NT_086827.

A 3411bp promoter sequence was amplified with Herculase polymerase (Stratagene) from human female genomic DNA using ATC GAT GAG CT/C AAA TAA AAT TCA CTT GCC CTA AGG TCC as forward and ATC GAT CTC GA/G TCC AAG GGC ACT TGA TTG GAT as reverse primers, where the SacI and XhoI restrictions sites are underlined respectively.

The conditions for the PCR reaction were as follows (50 μ L reaction): 3% DMSO, 2.5U Herculanase polymerase in buffer, 0.4 μ M of each primer, 100ng genomic DNA (Novagen), 0.2mM each dNTP. The thermocycler cycle was:

1x (94C, 3minutes) for initial denaturation;

35x {(94C, 45sec); (66C, 45sec); (72C, 4min30)} for amplification ;

1x (72C, 10min) for final elongation.

The -3297/+113hHL amplified fragment was directly subcloned into the pCR-BluntII-TOPO (Zero-Blunt TOPO PCR cloning kit from Invitrogen) and the amplified fragment was cut out by SacI and XhoI double digestion (NcoI, which does not cut the insert but cuts the vector twice, was also added to distinguish the insert from the vector), ran on a gel and gel extracted using the QIAquick gel extraction kit. The pGL3 basic was also digested with SacI and XhoI and dephosphorylated before it was run on a gel and gel extracted. The insert was then cloned into the MCS of the pGL3basic using sticky end ligation between the SacI and XhoI sites.

The sequence analysis described is in near complete agreement with the sequence of Oka et al. (59) with the exception of an additional T insertion at -140 (5T in a row in the sequence described here, versus 4 only reported in the Oka sequence). This T deletion in the Oka sequence also does not exist in the Ameis sequence. The numbering of the hHL promoter refers to the nomenclature of Oka et al (59) adjusted to the -140 insertion. The current sequence represents the “wild-type” haplotype at each of the 5 base pairs defining the common -514C/T polymorphism. The sequence differs from other reported sequences (accession numbers X58779 and M35425) (57) by the following point mutations: -708A→C, -709A→C, G insertion at -785, -1038 G→C, -1186 T deletion, -1330G→C. This -3297/+113hHL promoter vector was used as a template to amplify all further constructs for similar cloning in between the SacI and XhoI sites of the MCS of the pGL3basic. All primer pairs used the same reverse primer containing the XhoI restriction site: CGATCTCGA/GCTTGGTAATTTCTGAAGCC and the following

forward primers: CGATGAGCT/CGCAGTTGGGGGCAGTAA (-117/+14),
CGATGAGCT/CTTGATTAATTTGGAAGCTCTGAC (-249/+14),
CGATGAGCT/CAACACCACTTTCCTGAGC (-373/+14),
CGATGAGCT/CCCTCTCAATGGGTCACTT (-572/+14),
CGATGAGCT/CTGGTCGCCTTTTCCCTACC (-684/+14),
CGATGAGCT/CGAACTCCTGACCTCGTGAT (-965/+14),
CGATGAGCT/CTGTGTCAATGCATAAAAGTCA (-1480/+14).

All deletion inserts and junctions were fully sequenced.

For PCR conditions, see Table 3-1.

Table 3-1: PCR conditions for the cloning of hHL promoter constructs

	Denaturation (1) 1x	Amplification (2) 30x unless specified	Elongation (3) 1x
-117/+14	94C, 3min	40x {(94C, 45sec), (59C, 45sec), (72C, 1min)}	72C, 10min
-373/+14	94C, 3min	(94C, 45sec), (61C±2, 45sec), (72C, 1min)	72C, 10min
-572/+14	94C, 3min	(94C, 45sec), (61C±2, 45sec), (72C, 1min)	72C, 10min
-684/+14	94C, 3min	(94C, 45sec), (61C±2, 45sec), (72C, 1min)	72C, 10min

The conditions for the above PCR reactions were as follows (50µL reaction): 2% DMSO, 5U Taq DNA polymerase (Gibco), 2.5mM MgCl₂, 0.4µM of each primer, 0.5ng of -3297/+113hHL promoter vector as template, 0.2mM each dNTP, 1X buffer (Gibco).

-249/+14	95C, 1min	(95C, 1min), (54C±4, 1min), (72C, 1min)	72C, 10min
-965/+14	95C, 1min	(95C, 1min), (55C, 1min), (72C, 4min)	72C, 10min
-1480/+14	95C, 1min	(95C, 1min), (55C, 1min), (72C, 4min)	72C, 10min

The conditions for the above PCR reactions were as follows (50µL reaction): 2% DMSO, 2.5U Pfu Turbo DNA polymerase (Stratagene), 0.4µM of each primer, 1ng of -3297/+113hHL promoter vector as template, 0.2mM each dNTP, 1X buffer including 2mM MgCl₂ (Stratagene).

3.2.7 hHL promoter activity analysis

Principle of the assay

In the Dual-Luciferase® Reporter Assay, the activities of firefly (*Photinus pyralis*) and Renilla (*Renilla reniformis*, also known as sea pansy) luciferases are measured sequentially from a single sample. Firefly and Renilla luciferases have dissimilar enzyme structures and substrate requirements. Photon emission from the firefly luciferase reporter is measured first through oxidation of beetle luciferin in the presence of ATP, Mg^{2+} and O_2 . Immediately after quantifying the firefly luminescence, the luminescent signal from the firefly reaction is quenched by at least a factor of 10^5 and simultaneously, the Renilla luciferase reaction utilizing O_2 and coelenterateluciferin (coelenterazine) is activated. The amount of light integrated over a certain period of time is proportional to the amount of luciferase reporter activity in the sample, which in turn reflects the activity of the promoter driving its expression.

Assay in 24-well plate format

The empty pGL3 basic (negative control) or each construct were transfected alone or cotransfected with the pRL-TK vector (internal control containing the Renilla luciferase gene and used for normalization of cell density and transfection efficiency) into hepatoma cells. Typically, cells were transfected in 24 well plates with 80-200ng/well total DNA, with a 10:1 molar ratio of experimental luciferase reporter to pRL-TK control vector respectively. Transfection was done in a total volume of 250 μ L serum-free DMEM or MEM in the presence of lipofectamine and Plus reagent (Gibco) according to the manufacturer's instructions. Three hours after transfection, the medium was replaced and the appropriate maintenance or treatment medium was added to the cells. The cells were then treated for the time indicated in each experiment until collection. Both firefly and Renilla luciferase activities from 24-well plate lysates were measured using the Dual luciferase system (Promega) according to the manufacturer's instructions. Briefly, cells were rinsed with phosphate-buffered saline, and the PBS was aspirated completely.

100µl of 1X passive lysis buffer (PLB - provided) was dispensed in each well and the plates were shaken at RT on a rocking platform for 15 minutes, then frozen at -80C for anywhere from a few minutes up to a couple days. Lysis buffer was indifferently transferred to eppendorf tubes and spun down or pipeted directly from the wells of the culture plate into the assay tube. For each sample, the assays for firefly luciferase activity and Renilla luciferase activity were performed sequentially using 10 seconds integration times for each reading. First, a blank reading was obtained with 100µl of LAR II in a 12x75mm borosilicate glass tube. A second firefly reading was obtained after addition of 20µl of cell lysate into the luminometer tube and pipetting up and down. When applicable, a third and last reading for renilla luciferase was obtained after addition of 100µl of Stop & Glo® Reagent and vortexing.

Assay in 96-well plate format

White-sided, clear bottom, tissue-culture-treated, polystyrene 96 well plates (Costar 3610) were collagen-coated (50µl/well at 25µg/µl), and exposed to UV light overnight. This step was critical to prevent cells (especially McA-RH7777) from peeling off upon multiple changes of medium and thus to ensure obtaining a nice monolayer culture at time of collection. McA-RH7777 or Fu5AH cells were plated in 100µl medium at 40,000 cells/well one day before transfection. When the medium was changed, care was taken to pipet on the side of the wells such as not to disturb the cell monolayer. Typically, McA/Fu5AH cells were transfected in 96 well plates with 25ng/well total DNA, with a 50:1 molar ratio of experimental luciferase reporter to phRL-TK control vectors respectively. No luminescence signal was detected in 96 well plates when the pRL-TK vector was used, such as transfection experiments in 96 well-plates required to switch to the phRL-TK vector (Promega) with much enhanced promoter activity over the pRL-TK, thereby enabling to get solid renilla values with the 96 well plate luminometer. Transfection was done in a total volume of 70µL MEM (no serum) in the presence of 0.5µl lipofectamine and 1µl Plus reagent (Gibco life technologies) according to the

manufacturer's instructions. Three hours after transfection, the medium was replaced and 100µl of the appropriate maintenance or treatment medium was added to the cells. The luciferase assay was performed 24 to 48 hours after transfection. Both firefly and Renilla luciferase activities from 96-well plate lysates were measured using the Dual-Glo™ Luciferase Assay System (Promega #E2920 / E2940) according to the manufacturer's instructions. Briefly, plates were removed from the incubator, the medium was aspirated, 50µl MEM was added back to each well, and the plates were equilibrated to room temperature. 50µl of Dual-Glo™ Luciferase Reagent was added to each well and mixed in by gentle shaking of the plate. After 10-30 minutes waiting time, a white polyester sealing-tape (Nunc 235305) was taped to the bottom of the plate, such as the white-bottomed plate now had lower cross-talk interference upon reading in the luminometer (emission slide out). Firefly luminescence was measured over an integration time set at 5 seconds per well, reading from the top. 50µl of Dual-Glo™ Stop & Glo® Reagent was then added to each well, mixed, and Renilla luminescence was measured after 10-30 minutes.

Calculations:

Results were expressed relative to the Renilla luciferase internal control.

$\text{Firefly}_{\text{net}} = \text{Firefly}_{\text{gross}} - (\text{average of } \sim 3 \text{ firefly readings from untransfected wells})$

or because firefly reading from untransfected wells is about 0 above background:

$\text{Firefly}_{\text{net}} = \text{Firefly}_{\text{gross}} - (\text{background count (LARII reading) for same tube})$

$\text{Renilla}_{\text{net}} = \text{Renilla}_{\text{gross}} - (\text{average of } \sim 3 \text{ Renilla readings from untransfected wells})$

$\text{Firefly/Renilla ratio} = \text{Firefly}_{\text{net. average}} / \text{Renilla}_{\text{net. Average}}$

3.2.8 Lowry-Bensadoun Protein Assay

Total protein concentration in samples was assayed using an adaptation by Bensadoun and Weinstein (176) of the original Lowry et al. procedure (177) to assay protein in the presence of interfering materials such as lipids.

3.2.9 Preparation of nuclear extracts

Nuclear extracts from HepG2 cells in 100mm dishes were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce 78833) according to the manufacturer's instructions. The protein content of the crude nuclear extracts was assessed by Lowry after a preliminary deoxycholate-trichloroacetic acid (TCA) precipitation.

3.2.10 Gel shift and supershift assays

Tissue culture

HepG2 cells were split into 100mm dishes. Upon 90% confluency, cells were treated with LPDS medium with or without a mix of 10µg/mL cholesterol, 1µg/mL 25-OH-cholesterol and 5µg/mL 58-035 or 37.5µM compactin/mevastatin. DMSO and ethanol were added as carrier controls as appropriate. The treatment medium was refreshed after 24 hours. After 48 hours of treatment, nuclear extracts were prepared as described above (3.2.9).

DNA probe preparation

Reverse phase cartridge purified complementary oligos (with 5'OH ends) were ordered to use as probes.

Table 3-2: Probes used in gel shift and supershift assays

Probe #	Probe location in hHL promoter	Forward primer sequence	mer
1	-14/+14	ggtctctttggcttcagaaattaccaag	28
2	-70/-48	gagagggttaattattaatgggca	23
3	-110/-95	ggggcagtaaagaaag	16
4	-252/-218	aagttgattaatttggaactctgacctggcccca	35
5	-317/-298	gcagccacgtggaagccacc	20
6	-520/-509	ttgacacggggg	12
7	-566/-546	aatgggtcacttggaagggc	21
8	-595/-578	gaagtgtgttactctag	18
9	-929/-906	ccaaagtgtgtgattacaggagt	24

5 pmoles single-stranded oligos were end-labeled with 20 pmoles [γ -³²P]-ATP (3000Ci/mmol, 10mCi/mL) and 10U T4 polynucleotide kinase (NEB) for 1 hour at 37C, and the reaction was stopped by addition of 50mM EDTA. Double-stranded probes were annealed by mixing equimolar amounts of complementary forward and reverse primers in water, and incubating at 95C for 2 minutes, followed by 30 minutes at 37C. Double-stranded probes were purified on a 10% nondenaturing polyacrylamide gel (1X TBE: 0.089M Tris base, 0.089M boric acid, 2mM EDTA, pH 8.0), which had been previously prerun for 30 min at 5V/cm. The band was localized by exposure to film, excised from the gel, and transferred to a microfuge tube. To elute the DNA from the gel, the band (1 volume) was crushed with a p1000 tip, and incubated with 2 volumes of elution buffer (10mM Tris, 50mM NaCl, 1mM EDTA) for 4 hours at 37C. The elution buffer was recovered and the DNA was submitted to an ethanol precipitation using Glycoblue (Ambion) as a carrier. Probes were resuspended in 250 μ L TE, pH 8.0 and stored at 4C for up to 3 weeks.

Binding reactions for mobility shift assays

Frozen nuclear extracts were thawed on ice. Binding reactions for regular/competition gel shift assays were assembled in the presence of gel shift binding buffer (50mM KCl, 10mM HEPES, pH 7.9, 0.1mM EDTA, 2.5mM phenylmethylsulfonyl fluoride (PMSF), 2 μ g poly(dI-dC)·poly(dI-dC) (Amersham#27-7880-01), 10% glycerol, 0.3 μ g/ μ L BSA), with 20,000 to 30,000cpm probe and with or without 10 μ g crude nuclear extract (diluted more than 10 fold), and/or 1pmole cold unlabelled double-stranded probe competitor as appropriate. The sequence of the non-specific competitor used was GAGCCCAGCATTTTTGTGCGCA. The 20 μ L reaction mixes were incubated at 10-15C for 20 minutes in a constant-temperature water bath before being run for about 70 minutes at 35mA on a 4% nondenaturing gel with 2.5% glycerol, using 0.5X TBE as a running buffer. Binding reactions for supershift assays were assembled in the presence of supershift binding buffer (0.1M NaCl, 10mM HEPES,

pH 7.9, 0.1mM EDTA, 2.5mM PMSF, 3mM MgCl₂, 2μg poly(dI-dC)·poly(dI-dC), 10% glycerol, 0.1μg/μL BSA) with or without 10μg crude nuclear extract (diluted more than 10 fold) and/or 2μg antibody as appropriate in a total volume of 18μL. Reactions were preincubated at 4C for 2 hours to allow antibody to antigen binding, after which 20,000 to 30,000cpm probe was added to a final volume of 20μL. Binding reactions were then incubated for an additional 20 minutes at 10-15C before being run on a gel. All gels were prerun for 30 to 60 min at 100V immediately prior to loading. Loading dye was not added to binding reactions in order to prevent potential denaturing of complexes. Gels were exposed to a phosphorimager screen at room temperature (~1-24 hours in general) for analysis.

Antibodies

The following polyclonal immunoglobulin G (IgG) antibodies were purchased from Santa Cruz:

rabbit xUSF1 (H-86), sc-8983; rabbit xSREBP1a/c (H-160), sc-8984X; goat xSREBP2 (N-19), sc-8151X; rabbit xHNF1α (H-140), sc-10791; rabbit xTFIID/TBP, (SI-1), sc-273X; rabbit HNF4α, (H-171), sc-8987X; rabbit xCOUP-TFI, (H-60), sc-28611X; rabbit xRXRα, (D-20), sc-553; rabbit xRXRα, (ΔN 197), sc-774; rabbit xSp1, (H-225), sc-14027; rabbit xSp3 (D-20), sc-644; goat xSF1 (E-18), sc-10976X; rabbit xc-Jun (H-79), sc-1694X.

Antibodies obtained from other sources were mouse IgG2b xPPARα, (Affinity BioReagents, MA1-822), as well as house-raised rabbit antisera from Dr. L. Kraus laboratory (Cornell University) against c-jun (Jun_{NT}, PROD 1/2/03) or c-fos (3rd bleed, 280-335). Rabbit IgG (Sigma I-5006), goat IgG (Sigma I-5256), and mouse IgG (Sigma I-5381) were all stored in PBS, 0.02% azide.

3.2.11 Co- immunoprecipitation

Co- immunoprecipitation of transcription factors from non-transfected cells

800µg nuclear extract (see 3.2.9) from treated cells (see 3.2.10) was diluted 1:10 in gel shift binding buffer (50mM KCl, 10mM HEPES, pH 7.9, 0.1mM EDTA, freshly added 2.5mM PMSF) with 0.3µg/µL BSA and incubated for 1 hour at 4C with 8µg of either xUSF1 antibody (sc-8983), xHNF1α (sc-10791), xHNF4α (sc-8987), or rabbit IgG (Sigma I-5006). 50µL protein G- Sepharose 50% slurry (pre-equilibrated in binding buffer) was added to each tube for incubation overnight at 4C with constant gentle shaking. On day 2, the tubes were spun down at 5000rpm for 3 minutes at 4C to pellet the protein G complexes, and the beads were washed 4 times for 5-10 minutes each at 4C with 1mL of fresh chilled 0.1% Nonidet-P-40 in PBS. The protein complexes were released with 60µL elution buffer (50mM Tris-base, 100mM NaCl , 1% Triton X-100, 0.3% CHAPS, 1% sodium deoxycholate, 10mM EDTA, pH 9.0) for 30 minutes at 37C, the tubes were spun down, and the supernatants were collected. This elution step was repeated once. The eluate pools were concentrated down to <50µL with Microcon YM-30 (Millipore #42409) concentrators, which was achieved in a few minutes. Laemmli buffer (with β-mercaptoethanol) was added to 1X final, the samples were boiled for 3-5 minutes, and ran on a 10% SDS-PAGE. The MagicMark XP Western Protein Standard (Invitrogen) was used as a standard. The proteins were transferred to PVDF Immobilon P membrane using semi dry transfer, and the membranes were blocked overnight at 4C in PBS-tween with 5% non-fat dry milk. On day 3, the membranes were incubated overnight at 4C in blocking buffer supplemented with 1µg/mL xSREBP1 (sc-8984X). On day 4, the membranes were rinsed and washed six times for 10 minutes with PBS-tween (at 4-25C), then were incubated for 2 hours at RT in blocking buffer supplemented with anti rabbit-horseradish peroxidase (HRP) conjugate (Sigma A-9169) at 1:50,000 (Pierce). The membranes were rinsed and washed 6 times for 6-10 minutes with PBS-

tween at 4-25C, and revelation was achieved with the West Pico Reagents (Pierce) according to the manufacturer's instructions.

Co- immunoprecipitation of transcription factors from nSREBP1a or nSREBP1a and USF1 transfected cells

HepG2 cells in 100mm dishes were transfected with 5µg/dish of pCMV-SREBP1a-460 or pEGFP-N1 (Clontech 6085-1), or 2.5µg/dish of each pcDNA3-USF1 and pCMV-SREBP1a-460 in MEM using the Lipofectamine Plus reagent (Gibco/Life technologies). After 4 hours, the medium was aspirated and replaced by fresh serum-containing medium. The growth medium was replaced after 24 hours. 43 hours after transfection, the neutral cysteine protease and proteasome inhibitor N-acetyl-leucyl-leucyl-norleucinal (ALLN) was pipeted into the growth medium at 15µg/mL (39µM) final. After an additional 5-hour incubation, the medium was aspirated and nuclear extracts were prepared from cells transfected with nSREBP1a only or nSREBP1a and USF1 as described in 3.2.9. 5mg nuclear extracts were pre-cleared by centrifuging at 14,000rpm for 10 minute at 4C. The supernatants were diluted 1:5 in binding buffer (10mM Tris, pH 7.4, 150mM NaCl, 0.1% Triton X-100, 2.5mM PMSF) with 0.3µg/µL BSA and incubated for 1 hour at 4C with 4µg antibody or IgG as described above. The overall procedure for co-immunoprecipitation was the same as described above with the following specifications: 80µL protein G Sepharose 50% slurry was added to each tube, the proteins were released with 80µL elution buffer twice, and after transfer the membranes were blocked for 2 hours at room temperature.

3.3 Results

3.3.1 Decreased rHL mRNA levels upon cholesterol repletion

To determine whether the decrease in synthesized rHL upon cholesterol treatment could be at least partially explained by a decrease in transcription of the HL gene, the levels of rHL mRNA levels after cholesterol treatment were assessed. In these experiments, quantification of rHL mRNA levels by real-time reverse-transcription

polymerase chain reaction was done either by using a standard curve (data not shown) or by normalizing to P0 as presented, yielding virtually identical results. After a 24-hour treatment with sterols, an ACAT inhibitor or a combination of both, rHL mRNA levels (relative to P0) were 94.4%, 75.0%, and 77.5% of control cells (on LPDS medium) respectively (Figure 3-1). Overall, these data show that rHL mRNA levels are modestly decreased upon sterol treatment for 24 hours, and remain low over the second day of treatment (Figure 3-1).

To determine when the decrease in rHL mRNA is first detectable, a time course of rHL mRNA levels during cholesterol repletion was performed. rHL mRNA levels were analyzed by real time PCR and transcript numbers were calculated from a standard curve. rHL mRNA levels were found not to be changed over the first four hours of cholesterol repletion, but decreased thereafter, to 80.1% and 49.0% of control levels at 24 and 48 hours respectively (Figure 3-2).

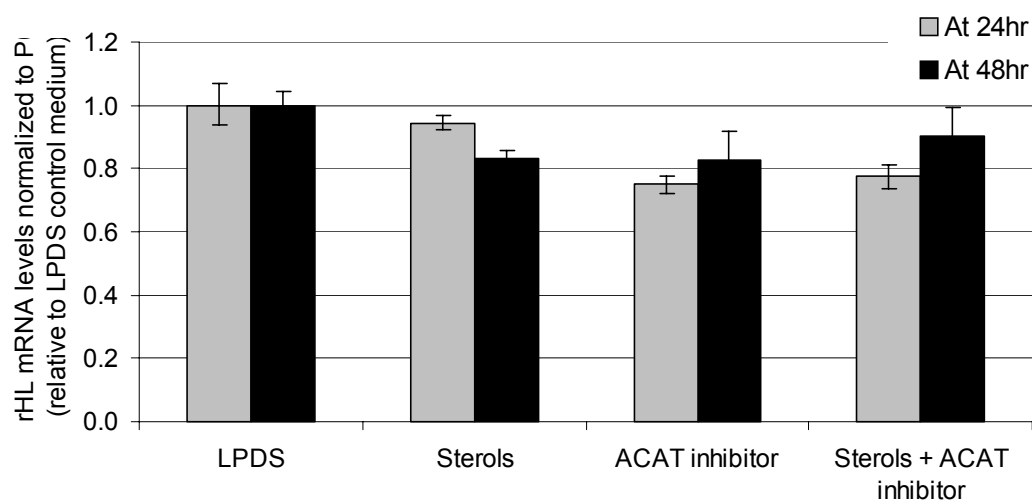


Figure 3-1 rHL mRNA levels are decreased upon sterol or 58-035 treatment

Fu5AH cells were split into 35 mm dishes. After 2 days, upon confluency, at t=0hr, triplicate wells were placed on 2mL/dish of either LPDS medium (10% LPDS, 2mM L-Glutamine in MEM) (LPDS), LPDS medium supplemented with 5µg/mL ACAT inhibitor 58-035 (ACAT inhibitor), LPDS medium supplemented with 10µg/mL cholesterol and 1µg/mL 25-OH-cholesterol (Sterols), or a combination of both (ACAT inhibitor + sterols). Ethanol and/or DMSO were added as carrier controls as appropriate. At t=24hr, the medium was refreshed on one subset of cells. For collection, cells were rinsed with PBS and collected in Trizol at t=24hr or 48hr as indicated. The Total RNA was extracted with Trizol (Invitrogen) according to the instructions of the manufacturer. mRNA was used as a template for real-time RT-PCR using TaqMan probes for rHL (custom-made Assay-by-design, Applied Biosystems) or ribosomal P0 (Assay-on-demand Rn00821065_g1 from Applied Biosystems). rHL mRNA levels were normalized to P0, and data were processed using the $\Delta\Delta C_t$ calculation method. Data are presented as percent change relative to control wells. Data for the 24 hour time point correspond to an average of two independent experiments.

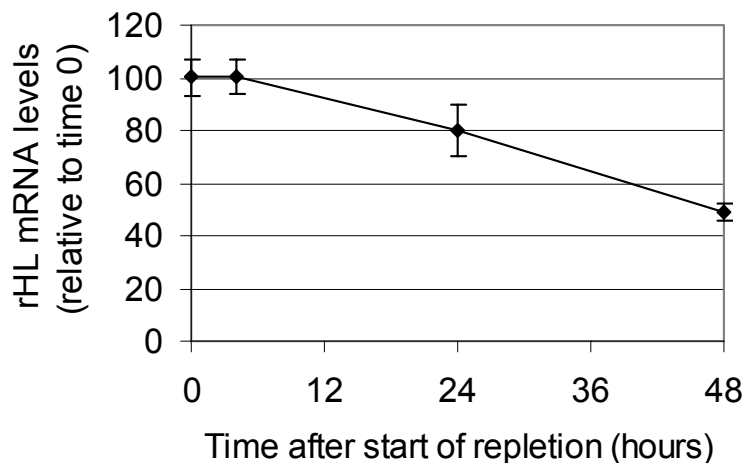


Figure 3-2 rHL mRNA levels upon cholesterol repletion

McA-RH7777 cells were split into collagen-coated 35mm dishes. At $t=-4$ hr, to deplete the cells of cholesterol, the cells were placed on 2mM methyl- β -CD in defatted medium. At $t=0$ hr, the medium was aspirated and replaced by 5mM methyl- β -CD 8:1 cholesterol (molar ratio) in defatted medium. This medium was refreshed at $t=24$ hr. At each collection time point, cells were rinsed with PBS and collected in Trizol. Total RNA was extracted with Trizol (Invitrogen) and further purified using RNeasy columns (Qiagen) according to the instructions of the manufacturers. mRNA was used as a template for real-time RT-PCR using a custom-made TaqMan assay for rHL (Assay-by-design, Applied Biosystems). Quantification of rHL mRNA levels was done using a standard curve and is expressed as percent change relative to time 0, when repletion of cholesterol began.

3.3.2 rHL mRNA degradation rate is not affected by sterol treatment

The observed decrease in rHL protein synthesis and decrease in steady-state mRNA levels could be due to either a decrease in the transcription of the HL gene and/or to an increase in the degradation rate of the HL mRNA. Figure 3-3 shows that the degradation rate of rHL mRNA appears to be unchanged by either sterol treatment or by 58-035 treatment over the first 24 hours of treatment, suggesting that the decrease in rHL steady-state mRNA levels in sterol-treated cells at 24 hours (Figure 3-1) is not due to a change in rHL mRNA stability. Again in this experiment, quantification of rHL mRNA levels was done either by using a standard curve (data not shown) or by normalizing to P0 as presented, yielding virtually identical results.

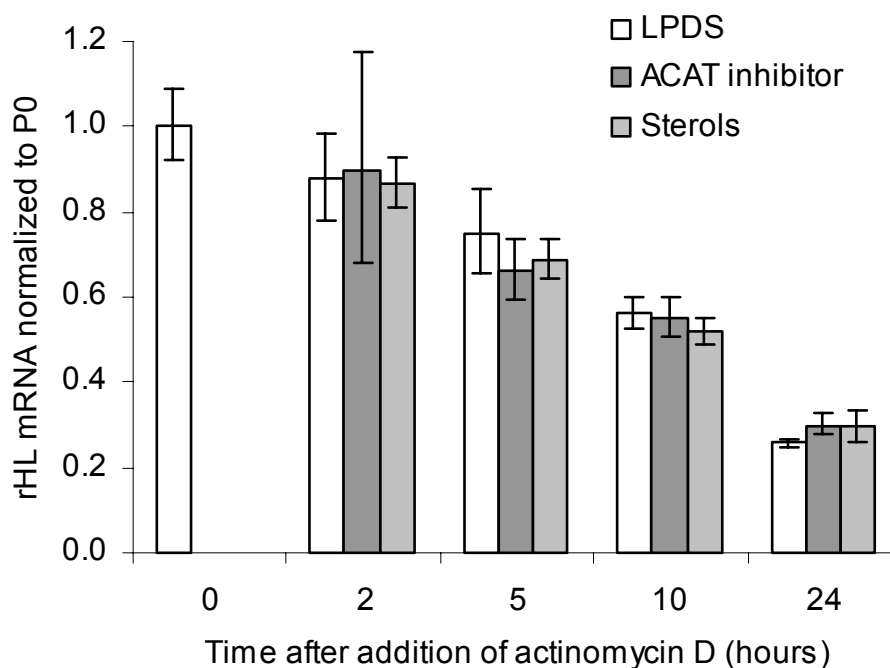


Figure 3-3 rHL mRNA degradation rate is unchanged upon sterol treatment

Two hours prior to the experiment, confluent Fu5AH cells in 35mm dishes were pre-incubated with LPDS medium (10% LPDS, 2mM L-Glutamine in MEM). At time t=0 hour, one set of cells was collected in Trizol for rHL mRNA baseline quantification, while other cells were placed on LPDS medium supplemented with 0.5 μ g/mL actinomycin D without (LPDS) or with 10 μ g/mL cholesterol and 1 μ g/mL 25-OH-cholesterol (sterols) or 2 μ g/mL 58-035 (ACAT inhibitor). Ethanol and DMSO were used as carrier controls as appropriate. Subsets of cells were collected in Trizol at the indicated time points (2, 5, 10 and 24 hours). Total mRNA was extracted and used as a template for real-time RT-PCR using TaqMan probes for rHL (custom-made Assay-by-design, Applied Biosystems) or ribosomal P0 (Assay-on-demand Rn00821065_g1 from Applied Biosystems). rHL mRNA levels were normalized to P0, and data were processed using the $\Delta\Delta$ Ct calculation method. Data are presented as fold change (average (n=3) \pm SD) relative to control wells at time 0.

3.3.3 The activity of a -1480/+14 human HL promoter fragment is unchanged by sterol or 58-035 treatment

Next, we examined transcriptional activity of the HL gene under sterol treatment. Whereas rat HL was studied in previous experiments involving analysis of protein levels (because the available rat HL ELISA is much more sensitive than for the human protein), the following studies on promoter regulation were performed using the human HL gene sequence available from NCBI.

In order to delineate the promoter sequence responsible for a decrease in the transcription of the HL gene, a series of hHL promoter deletion constructs were cloned upstream of a luciferase reporter gene. The deletion constructs spanned the hHL promoter from -1480bp to +14. None of the constructs was found to be responsive to sterol or 58-035 treatments under conditions where rHL mRNA is known to be repressed (Figure 3-4). Therefore, the effect of sterols or free cholesterol on the hHL promoter is not obvious, a result which was confirmed in multiple experiments in rat or human cells. Despite the lack of effect on the hHL promoter, these sterol treatments were effective at downregulating mature nSREBP transcriptional activity as shown by a decrease in the promoter activity of the pSRE-luc vector, where activity is driven by two sterol responsive elements (SREs) (see Figure 3-5). Because nuclear SREBP1c is very inefficient at activating an SRE, the activity of the pSRE-luc reflects the combined levels of mature SREBP1a and 2 isoforms in the nucleus (Table 1-1). Thus, sterol treatments resulted in a decrease in combined levels of nuclear SREBP1a and 2. Meanwhile, nuclear SREBP1c levels should have increased (73;126).

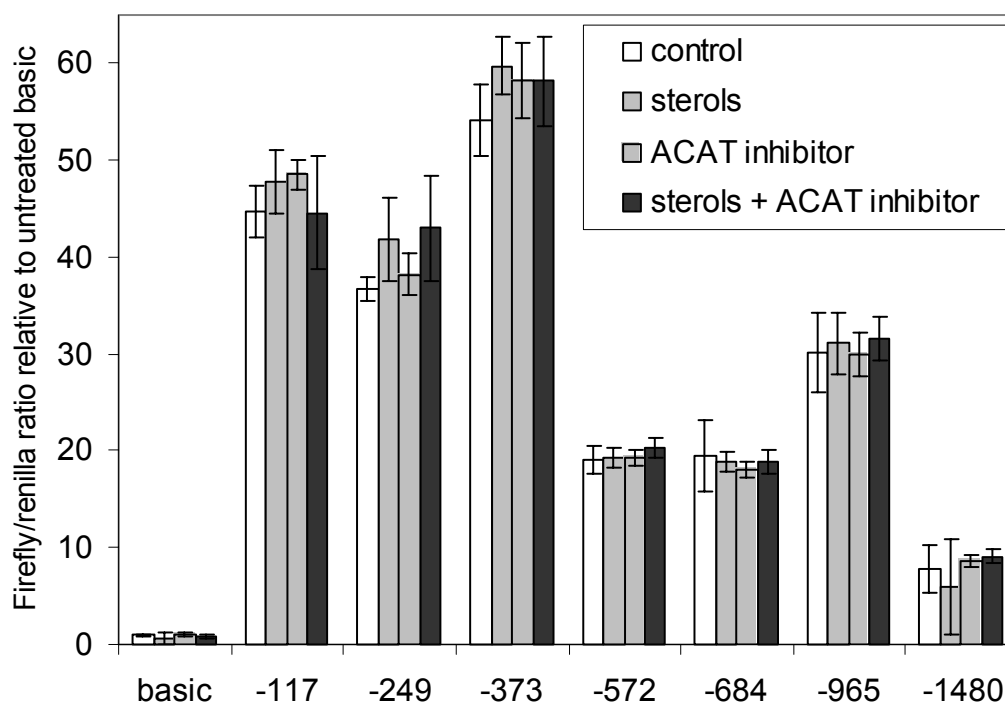


Figure 3-4 The activity of a -1480/+14 human HL promoter fragment is unchanged upon sterol or 58-035 treatment

Fu5AH cells were split into collagen-coated 96 well plates at a density of 40,000 cells/well. Two days later, at 95% confluency, at $t=0$ hr, each well was cotransfected in MEM with 2 vectors amounting a total of 25ng/well DNA using the Lipofectamine Plus Reagent (Gibco). phRL-TK (used for normalization of cell number and transfection efficiency) and pGL3 basic constructs (each containing the firefly luciferase gene under the control of different lengths of the hHL promoter) were added at a molar ratio of 1(phRL-TK):50(construct). At $t=3$ hr, the transfection medium was aspirated and replaced by 0.1mL fresh LPDS medium without (control) or with 5 μ g/mL 58-035 (ACAT inhibitor), or a mix of 10 μ g/mL cholesterol and 1 μ g/mL 25-OH-cholesterol (sterols), or a combination of both (sterols + ACAT inhibitor). Ethanol or DMSO were added as carrier controls as appropriate. At $t=24$ hr, the medium was aspirated and 50 μ L/well MEM was added. The luciferase assay was then performed according to the instructions of the manufacturer (Dual-Glo luciferase assay system, Promega). All values correspond to the ratio of the firefly luciferase activity to the renilla luciferase activity (both corrected for untransfected cells background) relative to the ratio obtained for the pGL3 basic empty vector in the absence of treatment (arbitrarily set at 1). Means \pm SD of 5-6 wells are shown. This experiment was representative of four similar experiments.

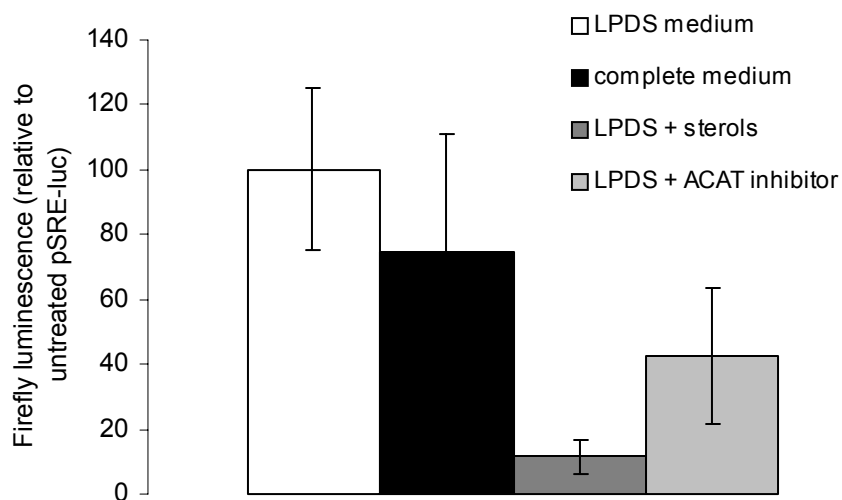


Figure 3-5: SREBP transcriptional activity is decreased in HepG2 cells treated with sterols or an ACAT inhibitor

At t=0hr, HepG2 cells at 90% confluency in 24 well plates were cotransfected in MEM using the Lipofectamine Plus Reagent (Gibco). Cells received 100ng/well pSRE-luc. At t=3hr, the transfection medium was replaced by 0.75mL LPDS medium with or without a mix of 10µg/mL cholesterol and 1µg/mL 25-OH-cholesterol (sterols) or 5µg/mL 58-035 (ACAT inhibitor), or complete medium as indicated. The medium was refreshed at 24 hours, and at t=48 hours cells were rinsed with PBS and lysed in 100µl/sample of 1X passive lysis buffer. The plates were shaken for 15 minutes and frozen down at -80C for enhanced lysis. The luciferase assay was performed according to the instructions of the manufacturer (Promega). Averages +/- SD, n=4.

3.3.4 nSREBP transfection decreases rHL mRNA levels

Because HL is known to increase the flux of cholesterol into liver cells and other HL-expressing cells (in the ovaries and adrenals), we sought to find out whether SREBP was involved in HL regulation as a part of its overall role in maintaining cholesterol cell homeostasis.

We first tried to see whether secreted HL levels would be altered upon nSREBP transfection. In McA-RH7777 cells maintained in complete medium such as endogenous nSREBP levels were maintained at a minimum, no change in HL secretion could be

detected 24 hours after transfection (data not shown). In the same experiment, USF transfection, a known activator of HL (80) also failed to upregulate HL secretion. In order to bypass any potential post-transcriptional regulation at the protein synthesis and secretion steps, we next examined mRNA and hHL promoter activity levels. Because the lack of an effect may be due to poor transfection efficiency, looking at hHL promoter activity levels where only transfected cells give a luminescence signal has the added advantage of increasing sensitivity.

nSREBP1a transfection in McA-RH7777 decreased levels of rHL mRNA compared to mock-transfected control cells after 15 hours (Figure 3-6). Because the experiment was conducted in defatted medium, endogenous mature nSREBP levels in control cells should have been high, which possibly could mask an effect of even greater amplitude. We reasoned that nSREBP could either affect rHL transcription directly or indirectly through another transcription factor. Alternatively, nSREBP could act by increasing cholesterol biosynthesis in the cells (upregulation of HMG-CoA reductase), which would lead to increased cell cholesterol levels, which in turn could downregulate HL, as was described previously. To test the latter hypothesis, we included a treatment with 50 μ M compactin in the same experiment. At this concentration, cholesterol biosynthesis should be efficiently suppressed, and although increased nSREBP levels would be expected to upregulate the expression of the LDLR, the cells could not take up cholesterol from the defatted medium, which does not provide a source of lipoproteins. 50 μ M mevalonate was also added in the treatment media, enough to provide for synthesis of non-sterol isoprenoids but not for sterol synthesis. nSREBP was found to downregulate rHL mRNA in the presence of compactin, and thus the effect of nSREBP appeared to be independent of cholesterol synthesis. The cholesterol effects, however, could be mediated by changes in nSREBP levels. Compactin alone was found to decrease HL mRNA levels, which could be explained by the fact that compactin itself is known to upregulate nSREBP (see Figure 3-10).

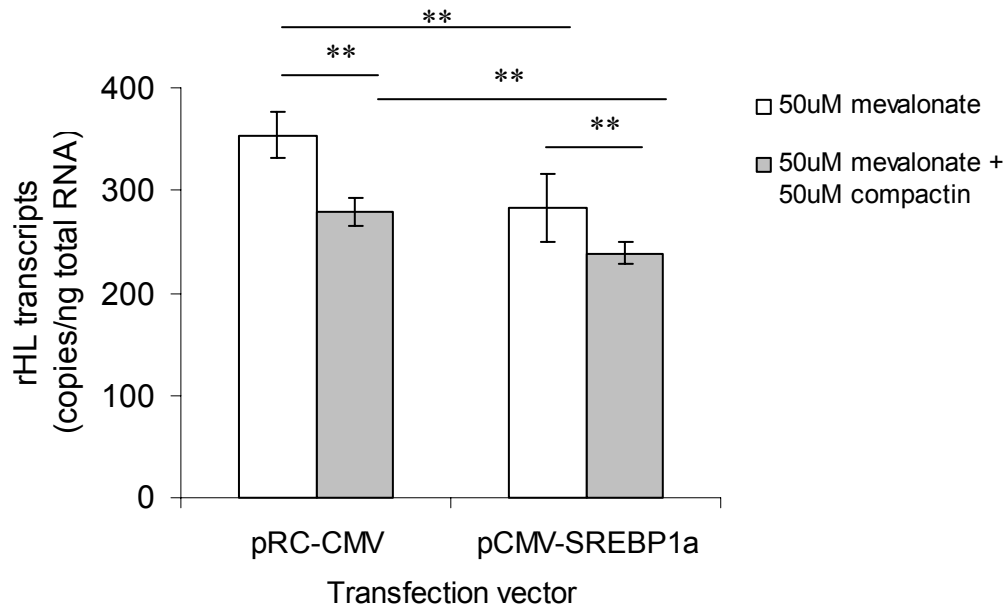


Figure 3-6 nSREBP1a and compactin decrease rHL mRNA levels

McA-RH7777 cells were split into collagen-coated 35mm dishes. At t=0hr, each well was transfected using the Lipofectamine Plus Reagent (Gibco) in DMEM with 0.8µg/well pCMV-SREBP1a-460 (ATCC) or pRc/CMV as a control. At t=3hr, the transfection medium was aspirated and replaced by 2mL defatted medium (containing 5% defatted FBS) with 50µM mevalonate and with or without 50µM compactin. At t=15hr, the cells were scraped and collected in the medium, transferred to eppendorf tubes and spun down. The medium was aspirated and the cells were rinsed with PBS. RNA was extracted using Trizol (Invitrogen) and further purified through RNeasy columns (Qiagen). rHL mRNA was quantified by real-time RT-PCR using a custom-designed TaqMan probe assay. **: statistical significance was assessed by 2-tailed unpaired Student's t-test (p<0.002).

3.3.5 The proximal human HL promoter fragment is activated by USF and inhibited by SREBP1a

Next we tried to see if the hHL promoter deletion constructs would be responsive to nSREBP1a transfection. Although we could not detect an effect of cholesterol on hHL promoter activity (Figure 3-4), if cholesterol could modify rHL transcription through SREBP, the direct overexpression of this transcription factor would magnify that effect.

In the presence of serum-containing medium in which endogenous nSREBP levels are expected to be low, overexpression of mature nSREBP1a was found to strongly inhibit all deletion constructs in McA-RH7777 cells, including the shortest -117/+14 fragment (Figure 3-7). Thus, a basal sequence necessary for inhibition appeared to be located within the -117/+14 sequence. Additionally, because the longer constructs appeared to have even stronger inhibition than the shorter ones, sequences further upstream in the promoter may cooperate in inhibiting transcription. Similarly, nSREBP1a transfection was found to downregulate hHL promoter activity in a human hepatoma HepG2 cell line (data not shown).

There are only a few examples of gene downregulation by SREBPs. In these cases, SREBP-induced downregulation of gene targets was proposed to be mediated either through binding to an SRE (146) or E-box (148) DNA sequence, or indirectly through binding to transcription factors such as Sp1(151) or LRH1(150) and inhibiting transactivation. In the case of HL, analysis of the proximal promoter failed to reveal the presence of an SRE. Conversely, sequence analysis revealed the presence of a few E-boxes in the HL promoter. One of these E-boxes is located at the common -514 polymorphism of the hHL promoter. The common C-514T mutation disrupts a CACGGG USF functional binding site by lowering the affinity of USF to the element by 2-4 fold (80). Functionally, there is a positive association between the CC patients with an intact USF binding site and higher HL activity levels (86). Interestingly, wild-type CC patients are also more responsive to statin therapy than TT patients or CT heterozygotes (44). Both nSREBPs and USFs are known to bind E-boxes. What's more, USF is known to induce HL promoter activity (80).

Together, this suggested that SREBP could inhibit HL promoter activity by competing with USF for binding to the HL promoter. In order to investigate the interaction between SREBPs and USFs in hepatoma cells, we tried to see whether nSREBP1a could reverse the USF-induced activation of HL by co-transfecting USF1

with increasing amounts of nSREBP1a. We found that both USF1 and USF2 could activate human HL promoter activity, and that this activation was effective with the shortest -117/+14 HL promoter construct available (Figure 3-8). Further, at stable levels of USF1, increasing levels of nSREBP1a decreased HL promoter activity of HL promoter constructs, which was obvious for the -373/+14 vector but also seemed true for the shorter -117/+14 construct. Similar experiments showed that the effect on the -249/+14 construct is the same as for the -373/+14 vector (data not shown). At high levels of nSREBP1 (a or c), HL promoter activity levels were low. This experiment was conducted in LPDS medium, where endogenous nSREBP levels should originally be high. In these conditions, a dominant negative USF1 construct lacking the DNA binding domain could also activate HL promoter activity. We repeated this experiment in HepG2 cells grown in complete medium, where endogenous nSREBP levels are expected to be low (Figure 3-9). Under these conditions, again, USFs could activate hHL promoter activity and this effect was repressed by co-transfection with increasing levels of nSREBP. Again, the effects were more marked for the longer -373/+14 vector than for the shorter -117/+14 vector. However, in these conditions, the dominant negative USF1 construct lacking the DNA binding domain could not activate HL promoter activity.

In this experiment, we also monitored the activity of the pSRE-luc vector to test for nSREBP transcriptional activity. As expected the SRE element was strongly activated upon transfection of nSREBP, such as the activity of the promoter was saturated even at the lowest levels of nSREBP tested. USF transfection did not affect pSRE-luc activity.

Overall, these experiments showed that downregulation of HL promoter activity by SREBP is mediated through an element within the -249 to +14 region. Because the -117/+14 vector yielded inconsistent data, we could not conclude whether some or all elements necessary for regulation lie within this region.

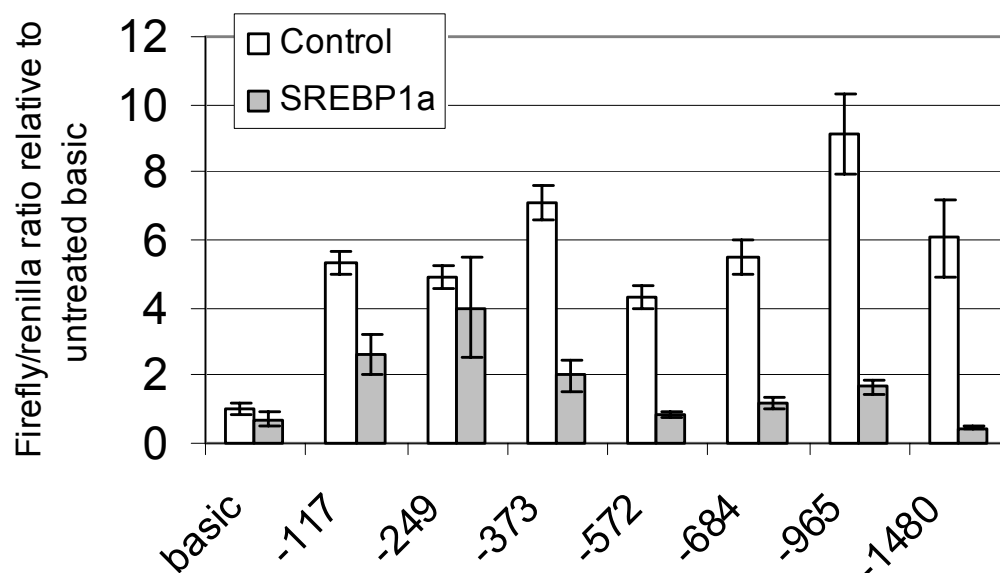


Figure 3-7 nSREBP1a decreases hHL promoter activity in McA-RH7777 cells

McA RH7777 cells were split into 24 well plates. At 90% confluency, at=0hr, each well was cotransfected in DMEM with 3 vectors amounting a total of 120ng/well DNA using the Lipofectamine Plus Reagent (Gibco). pRL-TK (used for normalization of cell number and transfection efficiency) and pGL3 basic constructs (each construct containing the firefly luciferase gene under the control of different lengths of the hHL promoter) were added at a total of 80ng/well at a molar ratio of 1(TK):10(constructor). In addition, cells received 40ng/well pCMV-SREBP1a-460 (ATCC) or pcDNA3 as a control. At t=3hr, the transfection medium was aspirated and replaced by 0.75mL fresh McA complete (serum-containing) medium. At t=24hr, the cells were scraped and collected in the medium, transferred to eppendorf tubes and spun down. The medium was aspirated and the cells were rinsed with PBS and spun down. The PBS was aspirated and the cells were resuspended in 100μl/sample of 1X passive lysis buffer (Promega) before being frozen down. The luciferase assay was performed according to the instructions of the manufacturer (Dual-luciferase reporter assay kit, Promega). All values correspond to the ratio of the firefly luciferase activity to the renilla luciferase activity (both corrected for background) relative to the ratio of the pGL3 basic empty vector in the absence of nSREBP1a transfection (normalized to 1). The data is an average +/- SD of 6-12 wells in 2 different experiments.

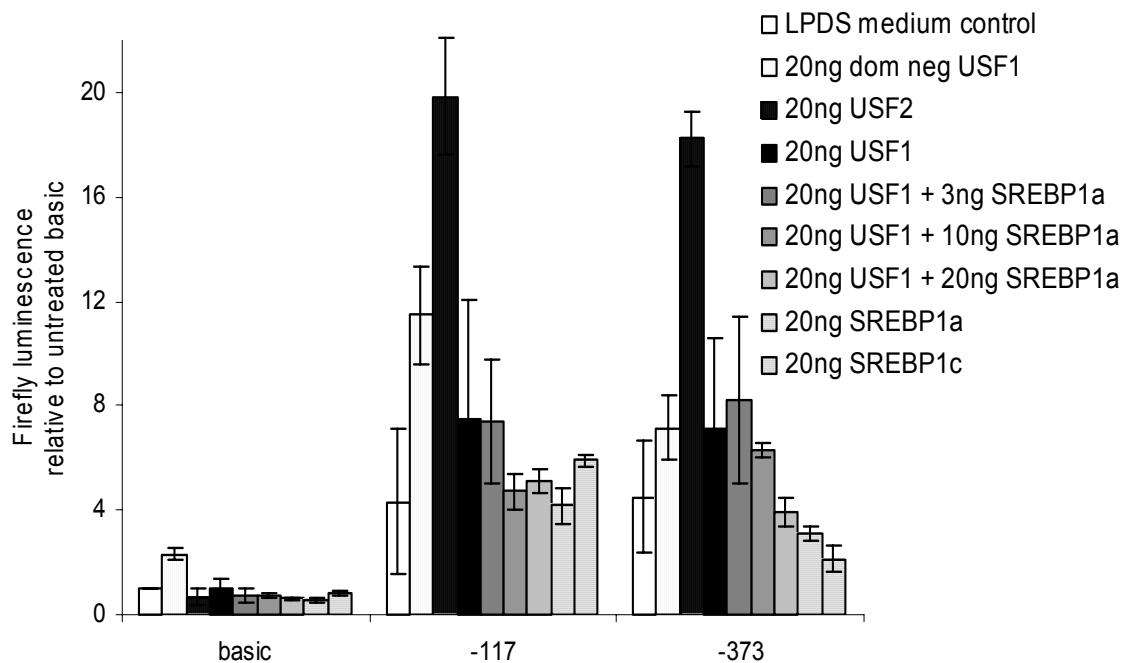


Figure 3-8: Wild-type and dominant negative USFs activate, and SREBP1 represses hHL promoter activity in LPDS medium

Fu5AH cells were split into 24 well plates. At 90% confluency, at=0hr, each well was cotransfected in MEM with a total of 190ng/well DNA using the Lipofectamine Plus Reagent (Gibco). pGL3 basic constructs (each construct containing the firefly luciferase gene under the control of different lengths of the human hepatic lipase promoter) were added at a total of 150ng/well. In addition, cells received up to 20ng/well of pCMV-SREBP1a-460 (ATCC), pCMV-SREBP1c, pcDNA3-USF1, pcDNA3-USF2, pcDNA3-dominant negative USF1, as indicated in the legend. The amount of DNA was adjusted to 190ng with pcDNA3 or pRc-CMV as appropriate. At t=3hr, the transfection medium was aspirated and replaced by 0.75mL fresh LPDS medium. Note that in such conditions, endogenous nSREBP levels would be expected to be high. At t=24hr, the medium was replaced, and at t=48 hours, cells were rinsed with PBS. The PBS was aspirated entirely, 100μl/sample of 1X passive lysis buffer (Promega) was added to each well, after which the plates were shaken at room temperature for 15 minutes and frozen down at -80C for enhanced lysis. The luciferase assay was performed according to the instructions of the manufacturer (Dual-luciferase reporter assay kit, Promega). All values correspond to the firefly luciferase activity corrected for background relative to the activity of the pGL3 basic empty vector in the absence of transcription factor transfection. Average +/- SD, n=3.

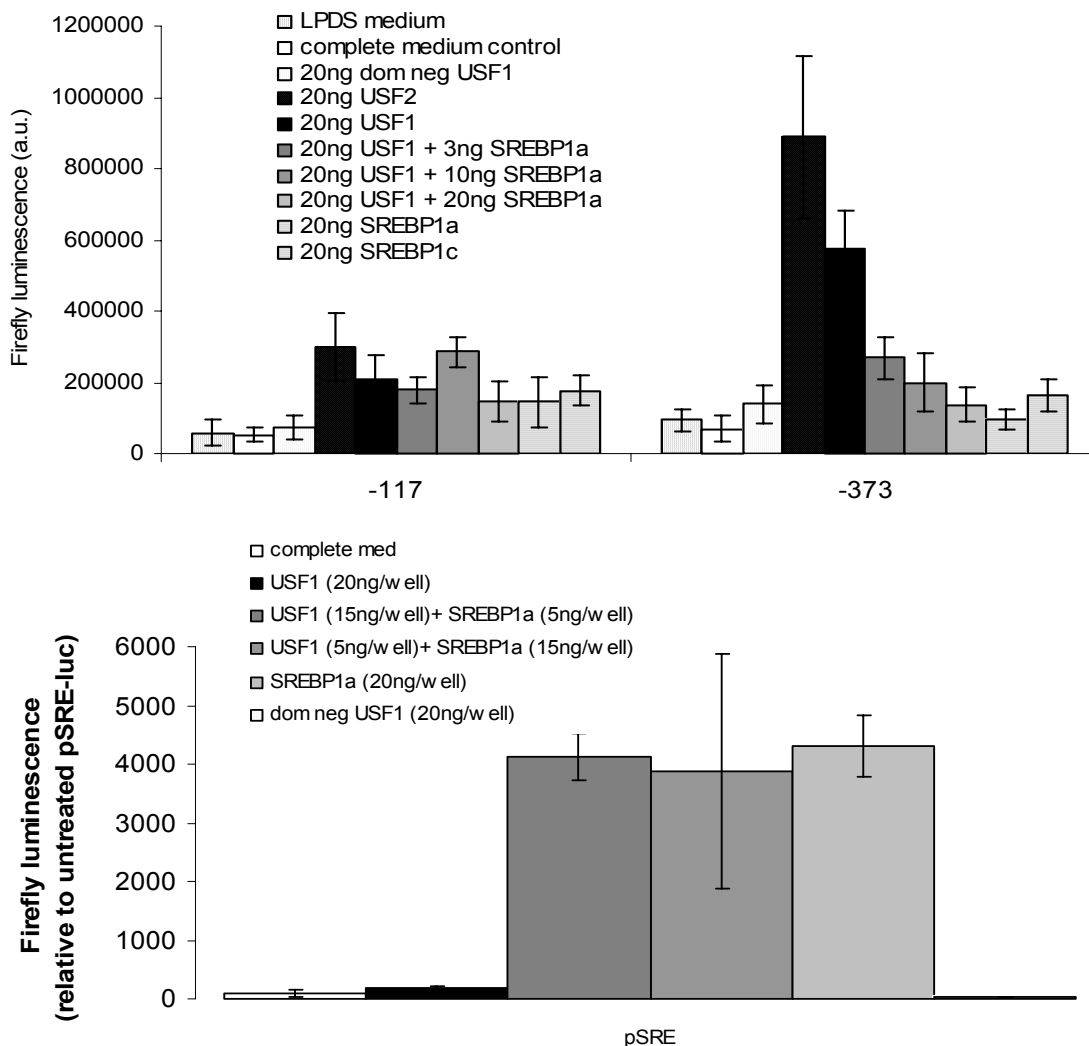


Figure 3-9: Wild-type USFs activate, and SREPB1 represses hHL promoter activity in complete medium

At t=0hr, HepG2 cells at 90% confluency in 24 well plates were cotransfected in MEM using the Lipofectamine Plus Reagent (Gibco). Cells received 150ng/well pGL3 hHL promoter constructs (top panel) or pSRE-luc (bottom panel) and up to 20ng/well each of pCMV-SREBP1a-460, pCMV-SREBP1c, pcDNA3-USF1, pcDNA3-USF2, or pFlag-dominant negative USF1, as indicated. The total amount of DNA was adjusted to 190ng/well with pcDNA3 or pRc-CMV as appropriate. At t=3hr, the transfection medium was replaced by 0.75mL fresh complete (10% FBS) medium, except for one set of cells which was treated with LPDS medium for comparison. The medium was refreshed at 24hrs, and at t=48 hours cells were rinsed with PBS and lysed in 100µl/sample of 1X passive lysis buffer. The plates were shaken for 15 minutes and frozen down at -80C for enhanced lysis. The luciferase assay was performed according to the instructions of the manufacturer (Promega). For HL promoter activity, values correspond to luciferase activity corrected for background (Average +/- SD, n=5). For pSRE-luc, values correspond to the ratio of luciferase activity for the indicated treatment to that in complete medium (Average +/- SD, n=4).

3.3.6 Compactin decreases hHL promoter activity while increasing nSREBP levels

As a further confirmation that increasing mature nSREBP levels yields a decrease in HL promoter activity, we treated cells with compactin, an inhibitor of HMG-CoA reductase. Compactin is known to increase nuclear SREBP2 levels (128). In HepG2 cells grown in LPDS medium, we confirmed that the 50 μ M compactin treatment increased pSRE-luc activity by 4.7 fold. In these conditions, there was a decrease in hHL promoter activity, at least for the shorter –117/+14 and –249/14 luciferase constructs (but apparently not the –373/+14 construct). These data are consistent with the nSREBP1a transfection data and suggest the presence of a responsive element within the –117/+14 region.

The trend for 20 μ M lovastatin to decrease HL levels was confirmed at the protein secretion level in McA-RH7777 cells, which were first cultured in defatted medium for 48 hours, and then treated for 48 hours in defatted medium with or without lovastatin (data not shown).

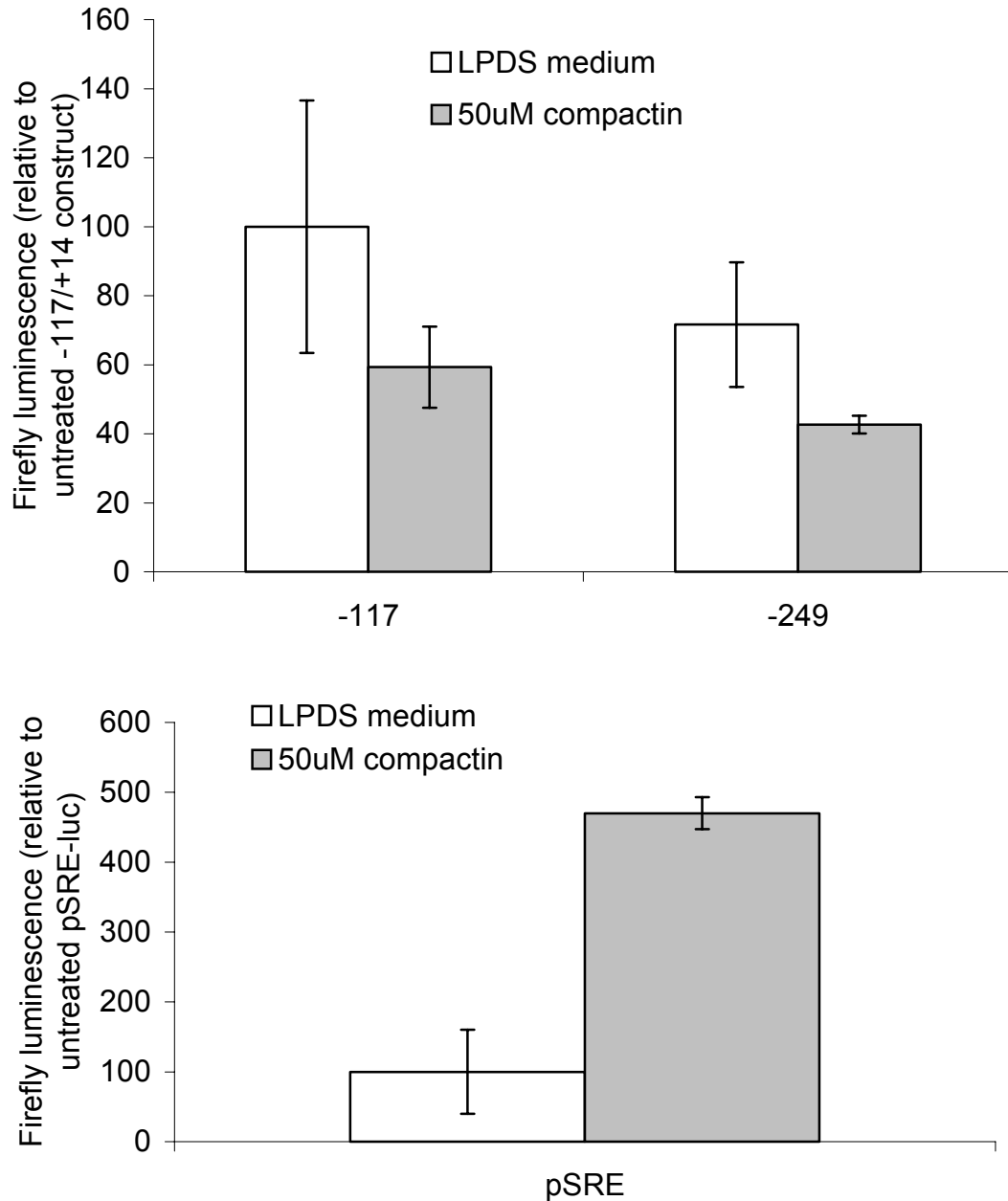


Figure 3-10: Compactin increases nSREBP transcriptional activity and decreases HL promoter activity in HepG2 cells cultured in LPDS medium

At t=0hr, HepG2 cells at 90% confluency in 24 well plates were cotransfected in MEM using the Lipofectamine Plus Reagent (Gibco). Cells received 100ng/well pGL3 hHL promoter constructs (top panel) or pSRE-luc (bottom panel). At t=3hr, the transfection medium was replaced by 0.75mL LPDS medium with or without 50μM compactin as indicated. The medium was refreshed at 24 hours, and at t=48 hours cells were rinsed with PBS and lysed in 100μl/sample of 1X passive lysis buffer. The plates were shaken for 15 minutes and frozen down at -80C for enhanced lysis. The luciferase assay was performed according to the instructions of the manufacturer (Promega). Averages +/- SD, n=4.

3.3.7 Gel shift analysis of the proximal hHL promoter

Having identified the proximal promoter (-249/+14) of hHL to be the minimal sequence required for regulation by USF and SREBP, we next turned to gel shift assays to try and identify factors that could mediate the response to USF and nSREBP transfection.

Within the -117/+14 hHL promoter fragment, we chose three probes (probes 1-3) covering the putative transcription factor binding sites within each of the three known DNase1 protected sites (see Appendix Q map). Within the -249/-117 region, we chose one probe (probe 4) using the same criteria as above. We also selected a few probes upstream of the critical -249/+14 region. Within the -373/-249 region, where there was no known DNase 1 protected sites, we chose one probe covering a putative site for USF, which also resembles an SREBP half-site (probe 5). For a similar reason, probe 9, which lies within a region where no DNase1 protection study was conducted, was selected because it covers a site which E-box sequence resembles SREBP binding sites. Thus, each of these two sites covers a putative binding sequence for sterol responsive transcription factors. Probe 6 was selected because it covers the -514 HL promoter polymorphism with its proposed functional USF site. Finally, probes 7 and 8 were selected because they cover known protected sites with putative binding sites for USF/steroidogenic factor/AP1 and AP1/forkhead related activator respectively. Altogether, within the proximal promoter, the selected set of probes covered putative binding sites within all known protected sites from both the Oka and the Hadzopoulou-Cladaras studies (59;60), with the exception of site C from the Hadzopoulou-Cladaras study at -158/-118, which we excluded because it was further upstream of the critical -117/+14 region, and because no known putative binding sites were identified using MatInspector (178), which would have made the identification of a binding factor even more difficult.

Probe 1, spanning the $-14/+14$ region of the hHL promoter, covered a protected site encompassing the minor and major transcription start sites. In this region, in gel shifts using nuclear extracts from control, compactin, or sterol-treated cells, we found no specific binding to the probe despite the presence of two non specific very faint bands (data not shown). Similarly, we found no binding to probe 3, spanning the $-110/-95$ region of the hHL promoter (data not shown). Probe 2, spanning the $-70/-48$ region of the hHL promoter, covered a known site for HNF1 binding (58). We were able to confirm that HNF1 could bind to this region in supershift assays (Figure 3-11). The intensity of the shifts using nuclear extracts from treated cells was decreased to $80.2\% \pm 4.7$ (sterols) and $69.3\% \pm 11.6$ (compactin) compared to shifts using extracts from control cells ($p < 0.02$ using Student's 2-tailed unpaired t-test) (Figure 3-11), suggesting that both treatments inhibit binding of HNF1 α to its response element.

Further upstream, probe 4, spanning the $-252/-218$ region of the hHL promoter, contained putative binding sites for a number of transcription factors. Incubating this probe with nuclear extract resulted in the appearance of two to three shifted bands (Figure 3-12). Of these, the 2 upper bands were not always distinguishable, and we therefore quantified them together, even though they may or may not be due to binding of the same factor(s). Antibodies against HNF1, COUP, Sp1, PPAR/RXR or Steroidogenic factor 1 could not supershift the lower band. We also found no evidence that they could supershift one of the upper bands, even though we could not rule out that possibility because of the presence of a single upper band in some of the supershift assays. An antibody against HNF4 α supershifted the single upper band. Additionally, in gel shifts using nuclear extracts from control or treated cells, we found that the intensity of the lower band and the upper band doublet was $84.2\% \pm 6.2$ and $80.7\% \pm 4.8$ (sterols) and $61.5\% \pm 10.5$ and $74.5\% \pm 10.3$ (compactin) respectively compared to control cells ($p < 0.01$ using Student's 2-tailed unpaired t-test). Therefore, both treatments inhibit binding of factors, in particular of HNF4 α , to this region.

Probe 5, spanning the -317/-298 region of the hHL promoter, covered a putative site for USF. This probe yielded one shifted band when incubated with nuclear extract from control or treated cells (Figure 3-13). The shifted band was completely supershifted with an antibody against USF1. Neither SREBP1 nor SREBP2 were found to directly bind to the probe, despite the presence of an SREBP1 half-site within the probe sequence. Gel shifts using nuclear extracts from control or treated cells showed that the intensity of the shift was $61.2\% \pm 20.1$ (sterols) and $70.5\% \pm 16.1$ (compactin) compared to control cells ($p < 0.01$ using Student's 2-tailed unpaired t-test) (Figure 3-13), suggesting that again, both treatments inhibit binding of USF1 to its response element.

Probe 6, spanning the -520/-509 region of the hHL promoter, was selected because it covers the -514 HL promoter polymorphism with its proposed functional USF site. This probe was not shifted using nuclear extracts from either control or treated cells.

Probe 7, spanning the -566/-546 region of the hHL promoter, was selected because it encompasses a known protected site with putative binding sites for USF/steroidogenic factor/AP1. Incubating this probe with nuclear extract resulted in the appearance of three shifted bands in gel shift assays (Figure 3-14). Of these, the 2 upper bands were quantified together for simplicity, yielding similar results as if they were quantified alone, while increasing the power. When using nuclear extracts from control or treated cells, we found that neither the intensity of the lower band nor of the upper band doublet were significantly changed $83.6\% \pm 11.1$ and $92.4\% \pm 23.8$ (sterols) and $84.2\% \pm 7.5$ and $100.3\% \pm 11.1$ (compactin) respectively compared to control cells ($p > 0.05$, using Student's 2-tailed unpaired t-test). Therefore, both treatments did not alter binding of factors to this region, and it appears unlikely that this region is involved in sterol regulation of HL (Figure 3-14). In a supershift assay using control crude extract in supershift binding buffer, surprisingly, only a single band was shifted. No evidence of binding was found using antibodies against HNF1 α , Sp1, Sp3, SREBP1, SREBP2 (data not shown), HNF4 α , COUP, AP1 (c-jun/c-fos) or Steroidogenic factor 1 (SF1) (Figure

3-14). An antibody against USF1 completely supershifted the single band (Figure 3-14), but it remains unclear which of the original three bands observed in gel shift assays was shifted with xUSF1.

Probe 8, spanning the -595/-578 region of the hHL promoter, was selected because it encompasses a known protected site with putative binding sites for AP1 and forkhead related activator 2. Incubating this probe with nuclear extract resulted in the appearance of two shifted bands in gel shift assays, which we quantified together for simplicity without a change in results (Figure 3-15). When using nuclear extracts from control or treated cells, we found that the intensity of band was not significantly changed $90.9\% \pm 29.8$ (sterols) and $130.7\% \pm 25.4$ (compactin) compared to control cells ($p > 0.05$, using Student's 2-tailed unpaired t-test) (Figure 3-15). In a supershift assay using control crude extract in supershift binding buffer, no evidence of binding was found using antibodies against HNF1 α , TBP, USF1, Sp1, Sp3, SREBP1, SREBP2, HNF4 α , COUP, or AP1 (c-jun/c-fos) (Figure 3-15). An obvious candidate for binding would be the forkhead related activator 2, but since this region does not appear to mediate the effects of sterols or compactin, we did not investigate this further.

Probe 9, spanning the -929/-906 region of the hHL promoter, lies within a region where no DNase1 protection study was conducted, but was selected because it covers a site which E-box sequence resembles SREBP binding sites. Incubating this probe with nuclear extract resulted in the appearance of two shifted bands in gel shift assays (Figure 3-16). When using nuclear extracts from control or treated cells, we found that the intensity of the lower and upper bands were $88.4\% \pm 3.8$ and $98.6\% \pm 7.3$ (sterols) and $104.8\% \pm 20.9$ and $109.4\% \pm 14.6$ (compactin) respectively compared to control cells. None of these changes were significant except for the lower band in the sterol treatment ($p < 0.01$, using Student's 2-tailed unpaired t-test). In a supershift assay using control crude extract in supershift binding buffer, no evidence of binding was found using antibodies against HNF1 α , USF1, Sp1, Sp3, SREBP1, SREBP2 (Figure 3-16).

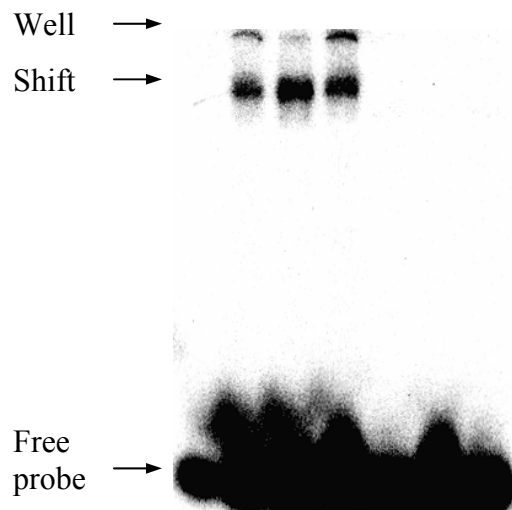
Overall, we were able to confirm HNF1 binding within the hHL promoter at -70/-48, and further identified binding sites for HNF4 and USF1 at -252/-218, and -317/-298 respectively. Binding of these factors was diminished using nuclear extracts from sterol or compactin treated cells. Further, we found that USF1 could bind to the -566/-546 region of the hHL promoter but that this binding was unresponsive to sterol or compactin treatment.

Figure 3-11 HNF1 α binds the –70/-48 region of the hHL promoter and this binding is decreased with extracts from sterol mix- or compactin-treated cells

Nuclear extracts were prepared from HepG2 cells treated for 48 hours in LPDS medium without (L) or with a mix of 10 μ g/mL cholesterol, 1 μ g/mL 25-OH-cholesterol and 5 μ g/mL 58-035 (S) or 37.5 μ M compactin (C). (A) Nuclear extracts were incubated for 20 minutes at 10-15C in gel shift binding buffer with 20,000cpm probe 2 (spanning the –70/-48 region of the hHL promoter) in the presence or absence of excess unlabelled specific (probe 2) competitor. The 20 μ L binding reactions were analyzed on a 5% nondenaturing gel in 0.5X TBE and the gel was exposed to a phosphorimager screen. (B) To allow antibody to antigen binding, nuclear extracts were pre-incubated for 2 hours at 4C in supershift binding buffer in the presence or absence of 2 μ g specific antibodies as indicated. After this preincubation, 20,000cpm probe 2 was added and the mix was incubated for an additional 20 minutes at 10-15C. The 20 μ L binding reactions were analyzed on a 4% nondenaturing gel in 0.5X TBE. (C and D) A similar gel-shift assay as in panel A was conducted in the presence or absence of excess unlabelled specific (probe 2) or non-specific (NS) competitor. Quadruplicate reactions were run from nuclear extracts from treated or non-treated cells and the density of the shifts was quantified in panel D.

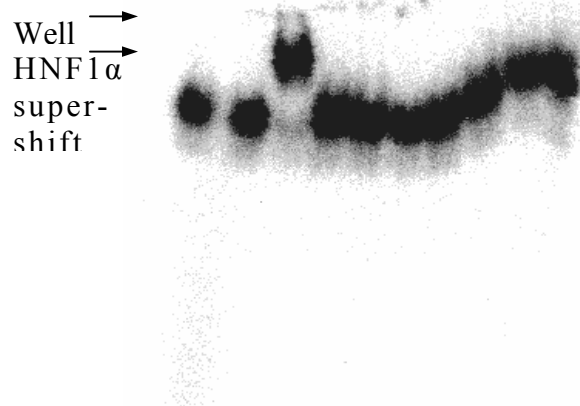
A.

Probe 2	+	+	+	+	+	+	+
10µg crude extract	-	L	S	C	L	S	C
Excess cold competitor	-	-	-	-	2	2	2



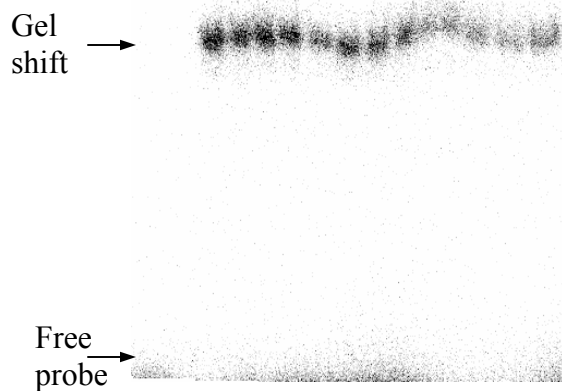
B.

Extr	-	L	L	L	L	L	L	L	L	L	L
	-	-	Rabbit IgG	xHNF1α	xTBP	xUSF1	xSREBP1	xSp1	xSp3	Goat IgG	xSREBP2



C.

Extract	-	L	L	L	S	C
Com- petitor	-	2	N S	-	-	-



D.

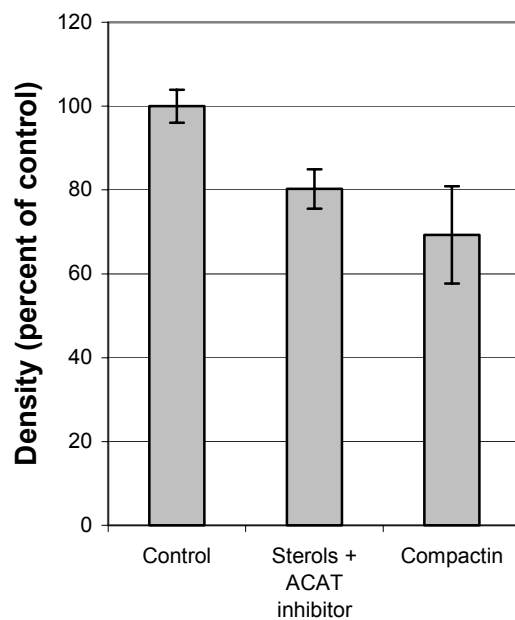


Figure 3-12 HNF4 α binds the –252/-218 region of the hHL promoter and this binding is decreased with extracts from sterol mix- or compactin-treated cells

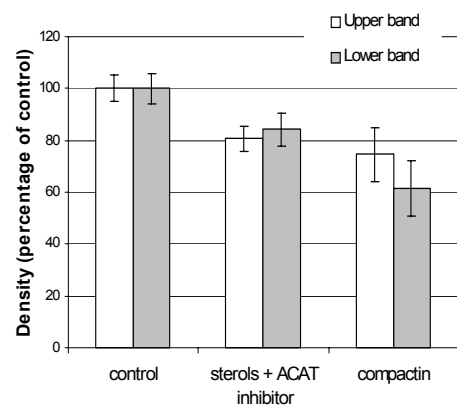
Nuclear extracts were prepared from HepG2 cells treated for 48 hours in LPDS medium without (L) or with a mix of 10 μ g/mL cholesterol, 1 μ g/mL 25-OH-cholesterol and 5 μ g/mL 58-035 (S) or 37.5 μ M compactin (C). (A) Nuclear extracts were incubated for 20 minutes at 10-15C in gel shift binding buffer with 20,000cpm probe 4 (spanning the –252/-218 region of the hHL promoter) in the presence or absence of excess unlabelled specific (probe 4) or non-specific (NS) competitor. The 20 μ L binding reactions were analyzed on a 4% nondenaturing gel in 0.5X TBE and the gel was exposed to a phosphorimager screen. (B) Quadruplicate reactions from nuclear extracts from treated or non-treated cells were run in a similar gel-shift assay as in panel A and the density of the shifts was quantified. (C-E) To allow antibody to antigen binding, nuclear extracts were pre-incubated for 2 hours at 4C in supershift binding buffer (C and D) or in gel shift binding buffer (E) in the presence or absence of 2 μ g specific antibodies as indicated. After this preincubation, 20,000-30,000cpm probe 4 was added and the mix was incubated for an additional 20 minutes at 10-15C. The 20 μ L binding reactions were analyzed on 4% nondenaturing gels in 0.5X TBE.

A.

Probe 4	+	+	+	+	+	+
10µg extr.	-	L	S	C	L	L
Competitor	-	-	-	-	4	NS



B.



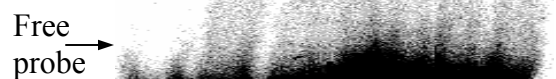
C.

Extract	-	L	L	L	L	L	L	L
	-	-	Rabbit IgG	xUSF1	xSREBP1	xSp3	xSREBP2	Goat IgG

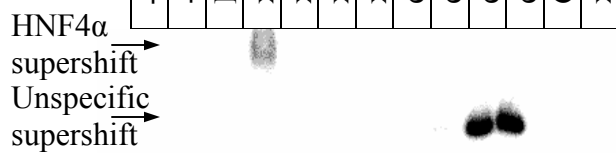


D.

Extract	-	L	L	L	L	L	L
	-	-	Rabbit IgG	xRXRα sc-553	xRXRα sc-774	Mouse IgG	xPPARα



E.													
Extr.	-	L	L	L	L	L	L	L	L	-	-	L	L
HNF4 α	-	-	Rabbit IgG	xHNF4 α	xCOUP	xHNF1 α	xSp1	c-fos antisera	c-jun antisera	c-fos antisera	c-jun antisera	Goat IgG	xSF1



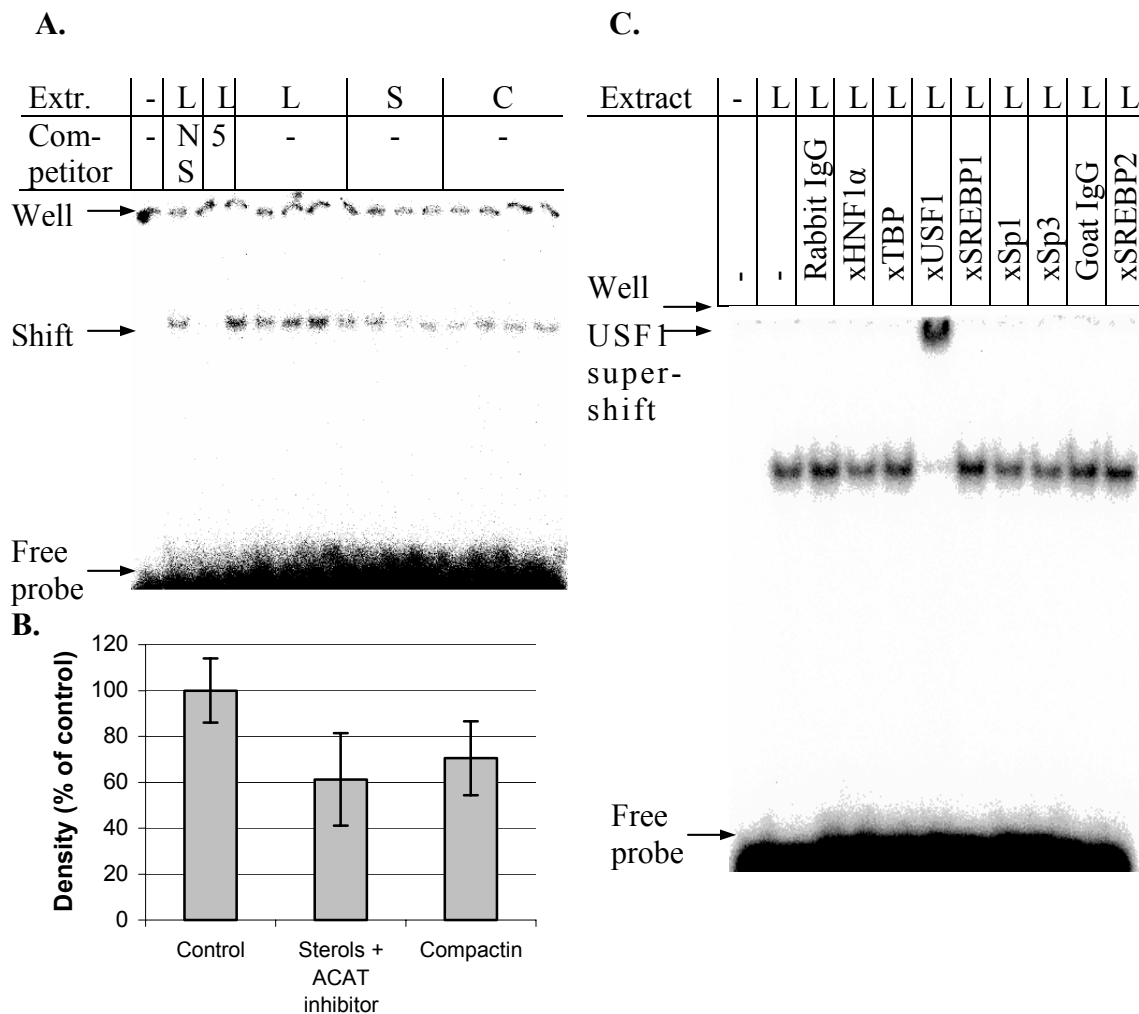


Figure 3-13 USF1 binds the -317/-298 region of the hHL promoter and this binding is decreased with extracts from sterol mix- or compactin-treated cells

Nuclear extracts were prepared from HepG2 cells treated for 48 hours in LPDS medium without (L) or with a mix of 10μg/mL cholesterol, 1μg/mL 25-OH-cholesterol and 5μg/mL 58-035 (S) or 37.5μM compactin (C). (A and B) Nuclear extracts were incubated for 20 minutes at 10-15°C in gel shift binding buffer with 30,000cpm probe 5 (spanning the -317/-298 region of the hHL promoter) in the presence or absence of excess unlabelled specific (probe 5) or non-specific (NS) competitor. The 20μL binding reactions were analyzed on a 4% nondenaturing gel in 0.5X TBE and the gel was exposed to a phosphorimager screen. Quadruplicate reactions were run from nuclear extracts from treated or non-treated cells and the density of the shifts was quantified in panel B. (C) To allow antibody to antigen binding, nuclear extracts were pre-incubated for 3 hours at 4°C in supershift binding buffer in the presence or absence of 2μg specific antibodies as indicated. After this preincubation, 20,000cpm probe 5 was added and the mix was incubated for an additional 20 minutes at 10-15°C. The 20μL binding reactions were analyzed on a 4% nondenaturing gel in 0.5X TBE.

Figure 3-14: USF1 binds the –566/-546 region of the hHL promoter and this binding is unresponsive to sterol or compactin treatment

Nuclear extracts were prepared from HepG2 cells treated for 48 hours in LPDS medium without (L) or with a mix of 10µg/mL cholesterol, 1µg/mL 25-OH-cholesterol and 5µg/mL 58-035 (S) or 37.5µM compactin (C). (A and B) Nuclear extracts were incubated for 20 minutes at 10-15C in gel shift binding buffer with 30,000cpm probe 7 (spanning the –566/-546 region of the hHL promoter) in the presence or absence of excess unlabelled specific (probe 7) or non-specific (NS) competitor. The 20µL binding reactions were analyzed on a 4% nondenaturing gel in 0.5X TBE and the gel was exposed to a phosphorimager screen. Quadruplicate reactions were run from nuclear extracts from treated or non-treated cells and the density of the shifts was quantified in panel B. (C and D) To allow antibody to antigen binding, nuclear extracts were pre-incubated for 2 hours at 4C in supershift binding buffer (C) or in gel-shift binding buffer (D) in the presence or absence of 2µg specific antibodies as indicated. After this preincubation, 30,000cpm probe 7 was added and the mix was incubated for an additional 20 minutes at 10-15C. The 20µL binding reactions were analyzed on a 4% nondenaturing gel in 0.5X TBE.

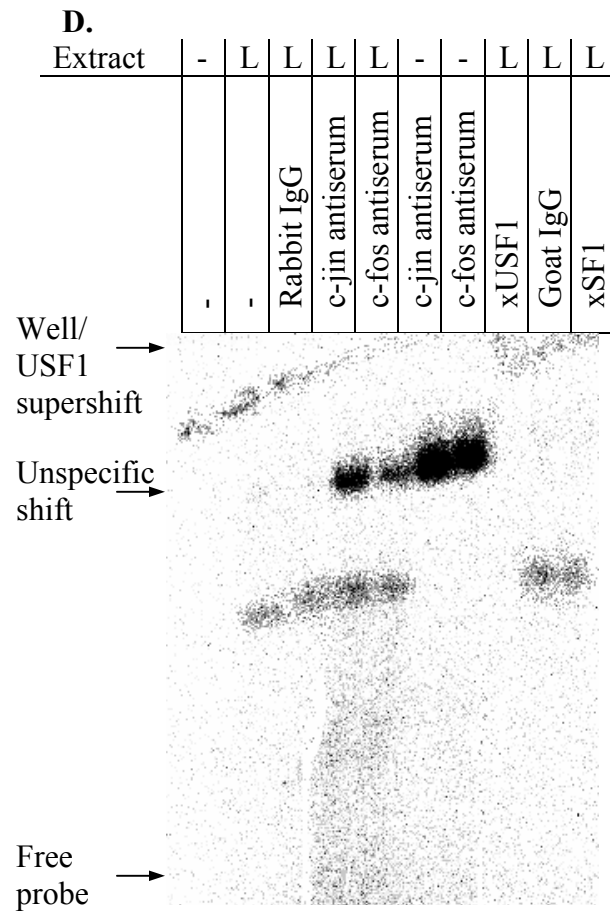
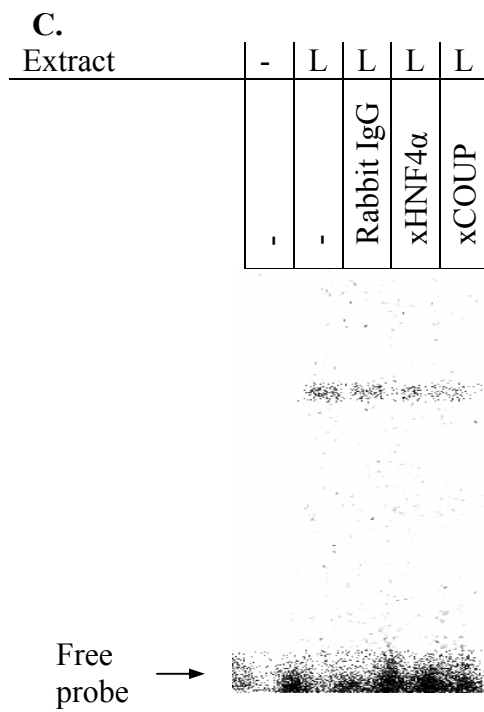
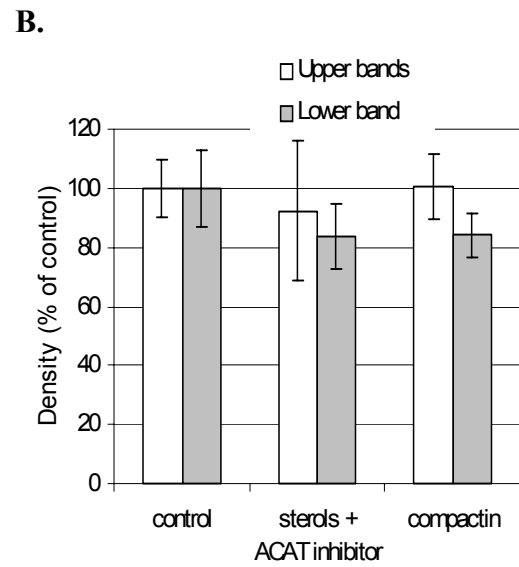
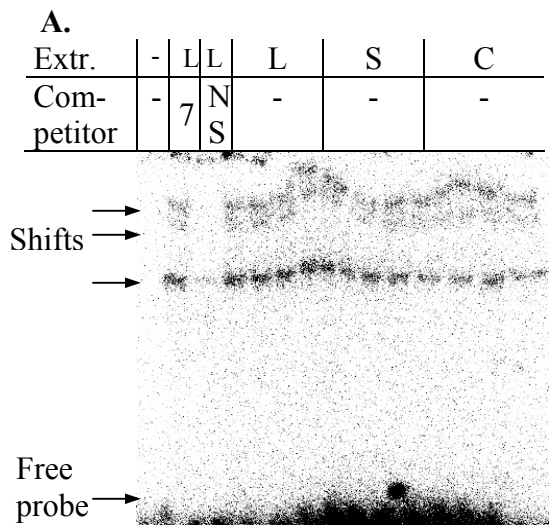
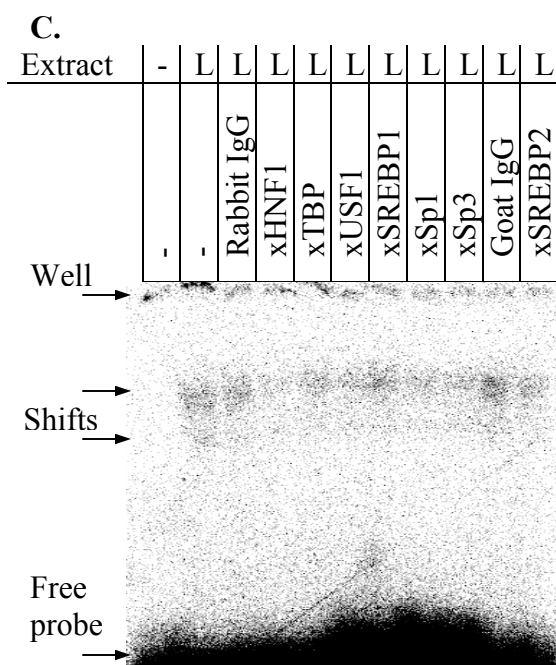
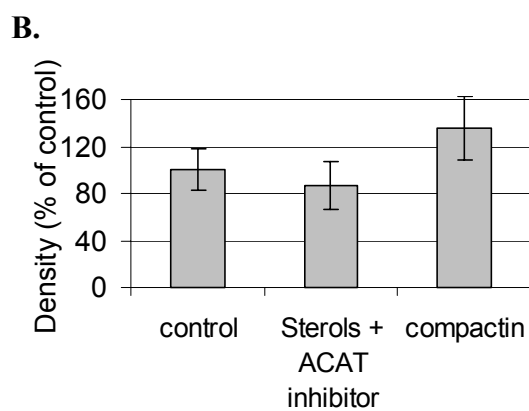
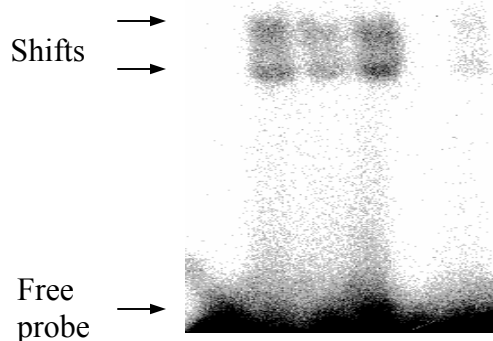


Figure 3-15: Unidentified factor(s) bind the –595/-578 region of the hHL promoter

Nuclear extracts were prepared from HepG2 cells treated for 48 hours in LPDS medium without (L) or with a mix of 10 μ g/mL cholesterol, 1 μ g/mL 25-OH-cholesterol and 5 μ g/mL 58-035 (S) or 37.5 μ M compactin (C). (A and B): Nuclear extracts were incubated for 20 minutes at 10-15C in gel shift binding buffer with 20,000cpm probe 8 (spanning the –595/-578 region of the hHL promoter) in the presence or absence of excess unlabelled specific (probe 8) or non-specific (NS) competitor. The 20 μ L binding reactions were analyzed on a 4% nondenaturing gel in 0.5X TBE and the gel was exposed to a phosphorimager screen. Quadruplicate reactions were run from nuclear extracts from treated or non-treated cells and the density of the shifts was quantified in panel B. (C, D and E): To allow antibody to antigen binding, nuclear extracts were pre-incubated for 2 hours at 4C in supershift binding buffer (C and D) or in gel-shift binding buffer (E) in the presence or absence of 2 μ g specific antibodies as indicated. After this preincubation, 20,000-30,000cpm probe 8 was added and the mix was incubated for an additional 20 minutes at 10-15C. The 20 μ L binding reactions were analyzed on a 4% nondenaturing gel in 0.5X TBE.

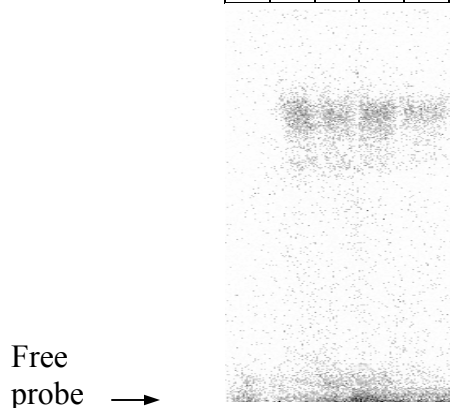
A.

Extract	-	L	S	C	L	L
Competitor	-	-	-	-	8	N S



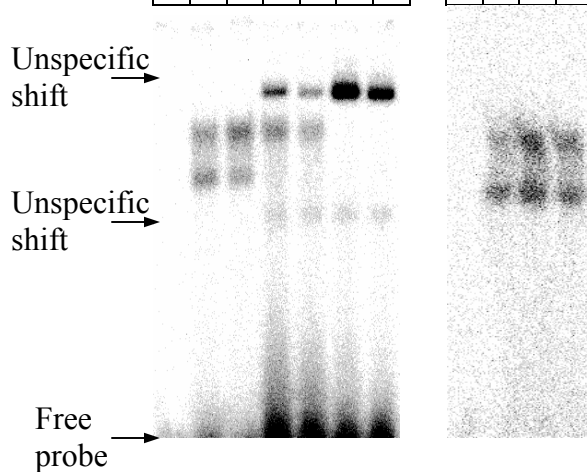
D.

Extract	-	L	L	L	L
	-	-	Rabbit IgG	xHNF4	xCOUP



E.

E xtract	-	L	L	L	L	-	-	-	L	L	L
	-	-	Rabbit IgG	c-jin antiserum	c-fos antiserum	c-jin antiserum	c-fos antiserum	-	-	Rabbit IgG	xc-jun



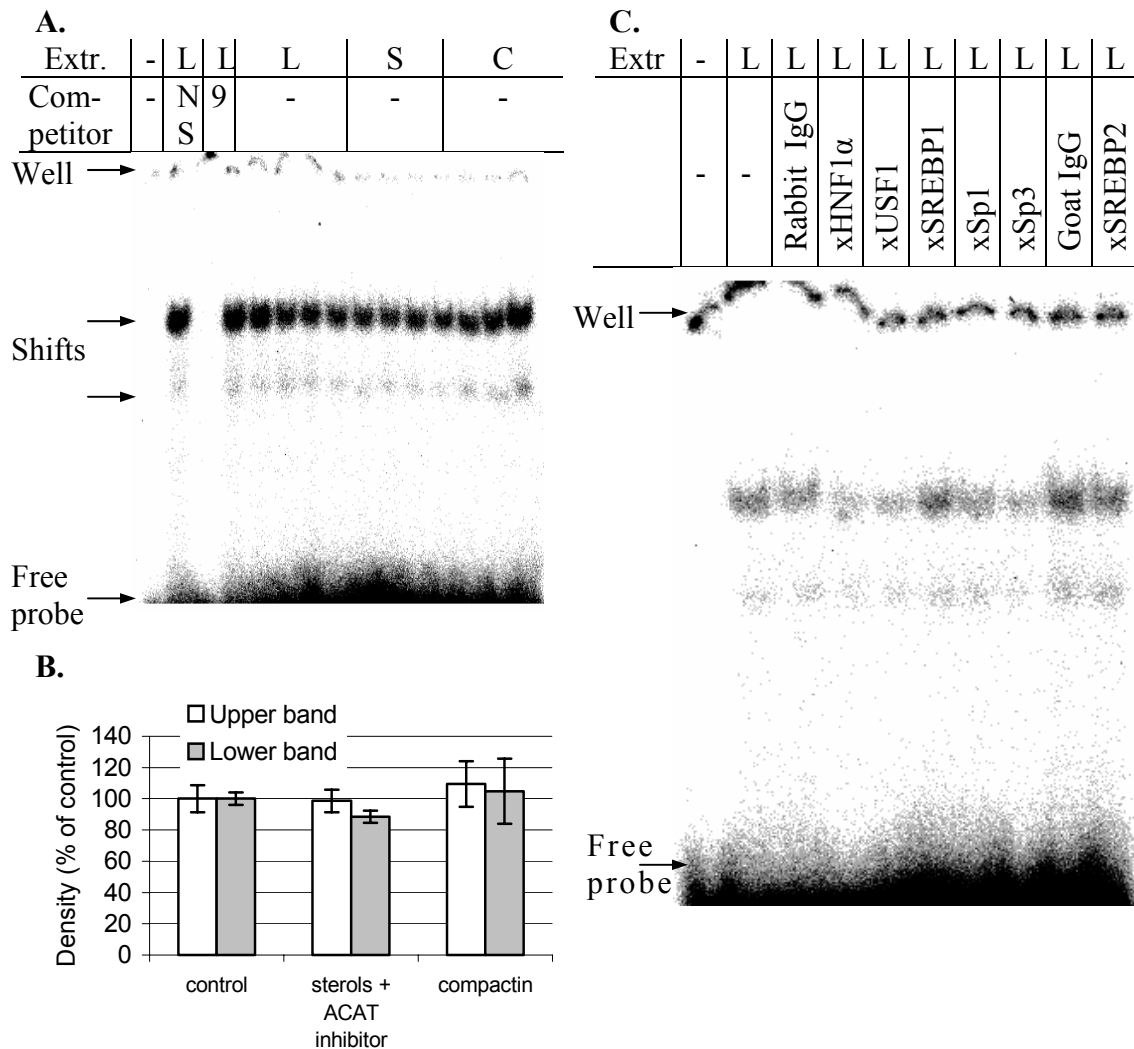


Figure 3-16 Unidentified factor(s) bind the -929/-906 region of the hHL promoter independently of sterol or compactin treatment

Nuclear extracts were prepared from HepG2 cells treated for 48 hours in LPDS medium without (L) or with a mix of 10 μ g/mL cholesterol, 1 μ g/mL 25-OH-cholesterol and 5 μ g/mL 58-035 (S) or 37.5 μ M compactin (C). (A and B) Nuclear extracts were incubated for 20 minutes at 10-15C in gel shift binding buffer with 30,000cpm probe 9 (spanning the -929/-906 region of the hHL promoter) in the presence or absence of excess unlabelled specific (probe 9) or non-specific (NS) competitor. The 20 μ L binding reactions were analyzed on a 4% nondenaturing gel in 0.5X TBE and the gel was exposed to a phosphorimager screen. Quadruplicate reactions were run from nuclear extracts from treated or non-treated cells and the density of the shifts was quantified in panel B. (C) To allow antibody to antigen binding, nuclear extracts were pre-incubated for 3 hours at 4C in supershift binding buffer in the presence or absence of 2 μ g specific antibodies as indicated. After this preincubation, 20,000cpm probe 9 was added and the mix was incubated for an additional 20 minutes at 10-15C. The 20 μ L binding reactions were analyzed on a 4% nondenaturing gel in 0.5X TBE.

Table 3-3: Recapitulative table of gel shift experiments

Probe location in hHL prom.	mer	Pro- tected sites (59;60)	Putative binding sites (Matinspector) (178)	Gel shift (specific unless otherwise specified)	Supershift	Sterol effect (p<0.01) compared to control	Compactin effect (p<0.01) compared to control
1-14/14	28	Hadz E1		non specific very light 2 bands	X	NA	NA
2-70/-48	23	Oka G	HNF1	HMW: 1 intense band LMW: 1 faint band which is the only one seen after heparin sepharose purification of extract	HNF1a, complete shift of strong HMW band	-20%	-30%
3-110/-95	16	Hadz B	Forkhead related activator 3	NONE	X	NA	NA
4-252/- 218	35	Hadz D	HNF1 /COUP /Sp1 /HNF4 /PPAR /RXR/steroidogenic factor 1	HMW: 1-2 bands of varying density LMW: 1 heavy band	HNF4a shifts middle/upper band(s)	2 upper bands: -19% ; lower band: -15%	2 upper bands: -25% ; lower band: -38%
5-317/- 298	20	no	USF /SREBP1	1 moderate band	USF1, complete	-39%	-29%
6-520/- 509	12	no	USF	NONE	X	NA	NA
7-566/- 546	21	Oka E	Ap1 /USF /steroidogenic factor 1	HMW: 2 bands LMW: 1 band Note: single faint band in supershift assays	USF1 shifts band	2 upper bands: no difference lower band: -16%	2 upper bands: no difference lower band: -15%
8-595/- 578	18	Oka D	Forkhead related activator 2 /API	2 bands	Non identified	no statistical difference	+36% (not significant)
9-929/- 906	24	NA	SREBP /Forkhead related activator 4	HMW: 1-2 bands LMW: fainter and sometimes undetectable band, which is the only one seen after heparin sepharose purification of extract	Non identified	HMW: no change lower band: -11%	no statistical difference

3.3.8 SREBP1 co-immunoprecipitation experiments

In the absence of binding of SREBP to the HL promoter, we sought to determine whether SREBP could inhibit HL through binding to known (USF1, HNF1 α) or putative (HNF4 α) HL activators.

We first attempted co-immunoprecipitation experiments using nuclear extracts prepared from HepG2 cells treated for 48 hours in LPDS medium without or with a mix of 10 μ g/mL cholesterol, 1 μ g/mL 25-OH-cholesterol and 5 μ g/mL 58-035 or 37.5 μ M compactin. 800 μ g nuclear extract were incubated for 1 hour at 4C in gel shift binding buffer (50mM KCl, 10mM HEPES, pH 7.9, 0.1mM EDTA, 2.5mM PMSF) with 0.3 μ g/ μ L BSA and 8 μ g of rabbit IgG or of an antibody against USF1, HNF1 α , or HNF4 α . 50 μ L protein G- Sepharose 50% slurry was added for an overnight incubation at 4C. The next day, the beads were washed with 0.1% Nonidet-P-40 in PBS and the protein complexes were released in elution buffer (50mM Tris-base, 100mM NaCl, 1% Triton X-100, 0.3% CHAPS, 1% sodium deoxycholate, 10mM EDTA, pH 9.0) for 30 minutes at 37C. The eluates were concentrated on Microcon YM-30 concentrators, run on a 10% SDS-PAGE along with MagicMark XP Western Protein Standard, and transferred to PVDF Immobilon P membrane using semi dry transfer, before a western blot for SREBP1 was performed. In these conditions (data not shown), with low endogenous levels of transcription factors, we were unable to detect an increase in co-immunoprecipitated SREBP1 in nuclear extracts first immunoprecipitated with xUSF1, xHNF1 α , or xHNF4 α compared to nuclear extracts mock immunoprecipitated with rabbit IgG.

Next, we decided to artificially increase the levels of transcription factors through transfection of either nSREBP1a alone, or nSREBP1a and USF1. In this experiment, in order to monitor transfection efficiency, we transfected one set of cells in the same conditions as for transcription factors with pEGFP-N1, a mammalian expression vector for the green fluorescent protein (GFP). Transfection efficiency with GFP vector was

very low, with far less than 2% of cells expressing GFP. Using nuclear extracts from cells transfected with nSREBP1a only, there was a high amount of noise on SREBP1 Western blots after a mock immunoprecipitation with rabbit IgG. This background noise prevented a conclusion to be drawn whether SREBP1 co-immunoprecipitated with USF1, HNF1 α , or HNF4 α (Figure 3-17). However, when using nuclear extracts from cells co-transfected with both nSREBP1a and USF1, it was clear that SREBP1a co-immunoprecipitated with USF1 (Figure 3-17). Thus, in overexpression experiments, we were able to show that SREBP1a can bind USF1, and possibly also HNF1 α and HNF4 α .

Magic marker	+	-	-	-	-	+	-	-	-	+
Transfected plasmids	-	S	S	S	S	-	S+ U	S+ U	-	-
First IP with	-	Rabbit IgG	xHNF1 α	xHNF4 α	xUSF1	-	Rabbit IgG	xUSF1	-	-

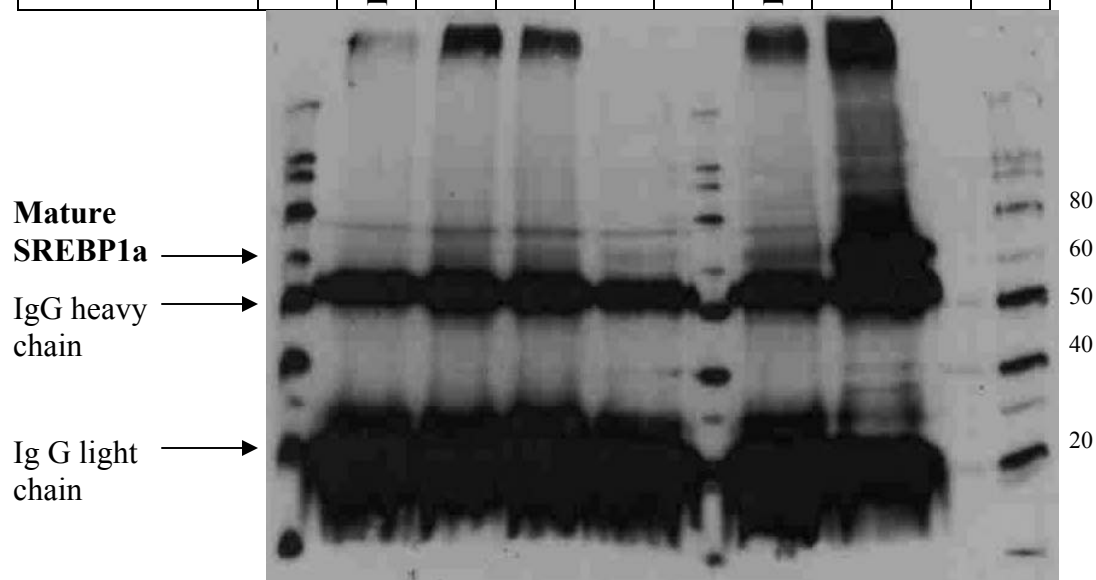


Figure 3-17 SREBP1 co-immunoprecipitates with USF1 in nSREBP1a and USF1 transfected cells

HepG2 cells were transfected with expression vectors for mature nSREBP1a (S) alone or in combination with USF1 (U), then were grown for 43 hours in complete medium before 15 μ g/mL N-acetyl-leucyl-leucyl-norleucinal (ALLN) was added to the growth medium for an additional 5-hour incubation. Nuclear extracts were prepared, and for each immunoprecipitation, 5mg nuclear extract was incubated for 1 hour at 4C in binding buffer (10mM Tris, pH 7.4, 150mM NaCl, 0.1% Triton X-100, 2.5mM PMSF) with 0.3 μ g/ μ L BSA and 4 μ g antibody (either xUSF1, xHNF1 α , or xHNF4 α) or rabbit IgG. 80 μ L protein G- Sepharose 50% slurry was added for an overnight incubation at 4C. The next day, the beads were washed with 0.1% Nonidet-P-40 in PBS and the protein complexes were released in elution buffer (50mM Tris-base, 100mM NaCl, 1% Triton X-100, 0.3% CHAPS, 1% sodium deoxycholate, 10mM EDTA, pH 9.0) for 30 minutes at 37C. The eluates were concentrated on Microcon YM-30 concentrators, run on a 10% SDS-PAGE along with MagicMark XP Western Protein Standard, and transferred to PVDF Immobilon P membrane using semi dry transfer before a western blot for SREBP1 was performed. Briefly, the membranes were blocked for 2 hours at RT in PBS-tween with 5% non-fat dry milk, incubated overnight at 4C in blocking buffer supplemented with 1 μ g/mL xSREBP1, rinsed and washed with PBS-tween, incubated for 2 hours at RT in blocking buffer supplemented with anti rabbit-HRP conjugate, rinsed and washed with PBS-tween at 4-25C. West Pico Reagents (Pierce) were used for revelation of the SREBP1 Western.

3.4 Discussion

The data presented in this chapter showed that nSREBPs inhibit the transcription of the HL gene and could be mediating the downregulation of HL under conditions where cholesterol is elevated and/or cholesterol synthesis is inhibited. Several activator binding sites within the hHL proximal promoter were identified through gel shift assays, but none for nSREBPs. Co-immunoprecipitation experiments suggest that inhibition of transcription may occur through interaction of SREBP with positive effectors of HL transcription such as USF1.

In parallel with the decrease in HL secreted protein mass and activity, rHL mRNA levels were decreased upon sterol treatment for 24 hours, and remained low over the second day of treatment (Figure 3-1). In the case of mRNA levels however, rHL mRNA levels were found not to be changed over the first four hours of cholesterol repletion, but only decreased thereafter, to 80.1% and 49.0% of control levels at 24 and 48 hours respectively (Figure 3-2). This timing difference suggests that the observed decrease in secreted HL reflects both an alteration of mRNA levels and regulation at the translational or post-translational level. The cause for the decrease in mRNA levels was further investigated in this chapter. The mechanism by which secretion of HL protein was decreased at stable mRNA levels was investigated in chapter 4.

The decrease in steady-state HL mRNA levels after 24 or 48 hours of treatment could be due to a decrease in transcription and/or an increase in mRNA degradation. Using actinomycin D to inhibit general transcription, we found that HL mRNA degradation rate was not changed (Figure 3-3), suggesting that the change in mRNA steady-state levels is instead due to a decrease in the transcription of the HL gene. In order to try and delineate the promoter sequence responsible for a decrease in the transcription of the HL gene, we created a series of hHL promoter deletion constructs spanning the hHL promoter from -1480bp to +14 and driving the expression of a luciferase reporter gene. Under conditions where rHL mRNA is known to be repressed, we consistently found that sterols or 58-035,

an ACAT inhibitor, had no effects on the activity of any of the promoter constructs (Figure 3-4). These sterol treatments were effective at downregulating mature nSREBP transcriptional activity as shown by a decrease in the promoter activity of the p-SRE-luc vector, which activity is driven by two SRE elements (Figure 3-5). All the data presented thus far showed a downregulation of rat HL while we used a human HL sequence for promoter experiments. Yet, this species variation was not expected to make a difference since hHL was previously reported to be repressed by mevalonic acid in HepG2 cells (74). The reason why sterols had no effect on hHL promoter activity in our assay remains unclear. Because direct transfection of nSREBP could decrease HL promoter activity, it is unlikely that our assay failed because of the lack of some HL promoter chromatin structure essential for repression. It is possible that sterols indeed have no effect on this portion of the promoter. However, this same portion of the promoter was found to be sensitive to nSREBP transfection in promoter activity assays, and using nuclear extracts from control or sterol treated cells in gel shift assays, we found that sterols affect the binding of some activators to probes within this region. We know that total nSREBP activity was decreased in our assay, but we could not quantify the activity or levels of each isoform specifically. nSREBP1a and 2 were both expected to decrease upon sterol addition, but the nSREBP1c isoform specifically should have increased (126). To which extent a decrease in 1a was balanced by an increase in 1c is unknown. If such compensation could have blunted the effect on HL promoter activity levels, it is unclear why sterols could nonetheless decrease HL mRNA and secretion levels under the same treatment conditions.

The pGL3 basic vector (without an inserted promoter) was previously found to have an E-box (5'-CGCGTG-3') in between the MluI and NheI restriction sites (179) that binds to SREBP1a and 1c. However, this problem was avoided in this study by cloning the hHL promoter fragments in between the SacI and XhoI sites, thereby deleting this E-box in the series of constructs used for the study of promoter regulation. Original

promoter activity assays with sterols (Figure 3-4) or nSREBP1a (Figure 3-7) treatments were done by normalizing firefly luciferase activity to renilla activity. The pRL-TK vector expresses Renilla luciferase under the control of the herpes simplex virus thymidine kinase promoter. This vector has been shown to vary upon treatment with adenoviral 12S E1A oncoprotein in Saos-2 cells (180) or dihydrotestosterone and dexamethasone in CV-1 cells (181), making it an inappropriate control for the study of these transcriptional regulators. Because this internal control was sometimes markedly responsive to sterol treatments, we tried to switch to pH β APr-1- β Gal (a β galactosidase expression vector driven by the human β actin promoter (182), gift from Dr. E. Keller, data not shown), which also showed to be responsive to sterol treatment. In the absence of an internal control which expression was not affected by the treatment, the promoter activity data was drawn from statistical analyses of un-normalized replicates in multiple experiments, yielding more reliable results. After confirming the previous results in this manner, we performed later assays with nSREBP1a and USF (Figure 3-8 and Figure 3-9) or compactin (Figure 3-10) using firefly constructs only.

Cholesterol is known to downregulate processing of SREBP1a and 2, which when mature activate genes involved in lipid metabolism by binding to SREs or SRE-like elements. Unlike lipoprotein lipase (62), HL has never been shown to date to be under the direct control of SREBPs. Yet, because HL expression is known to influence cell cholesterol levels, we sought to find out whether SREBP was involved in HL regulation as a part of its overall role in maintaining cholesterol cell homeostasis. If cholesterol could modify rHL transcription through SREBP, the direct overexpression of this transcription factor would magnify that effect. We failed to see a change in HL protein secretion 24 hours after nSREBP1a transfection in McA-RH7777 cells maintained in complete medium (data not shown). In the same experiment, USF transfection, a known activator of HL (80)(76) also failed to upregulate HL secretion, which suggested that the sensitivity in this assay may be too low to detect a change even if present. This is not a

surprising finding since transfection efficiency in hepatoma cells is very low (see 3.3.8) and most cells do not express the exogenous DNA. In order to bypass potential post-transcriptional regulation, we next examined mRNA and hHL promoter activity levels. Promoter activity assays in particular provide increased sensitivity since only transfected cells give a luminescence signal. nSREBP1a transfection in McA-RH7777 decreased levels of rHL mRNA compared to mock-transfected control cells after 15 hours (Figure 3-6). Additionally, nSREBP was found to downregulate rHL mRNA in defatted medium with compactin, conditions where the nSREBP-induced increase in cholesterol biosynthesis and lipoprotein uptake was blocked. Thus, the inhibitory effect of SREBP appeared to be independent of total cholesterol levels. Overexpression of mature nSREBP1a in complete medium was found to strongly inhibit all deletion constructs in rat (Figure 3-7) or human hepatoma cells, including the shortest -117/+14 fragment. Sequences further upstream in the promoter may cooperate to potentiate the inhibition of transcription. Compactin, which increased pSRE-luc activity by 4.7 fold in our experiments (Figure 3-10), also led to a decrease in HL mRNA and in the activity of the -117/+14 hHL promoter fragment. Further, statins also lead to a decrease in the secretion of HL mass. Taken together, we found a consistent inverse relationship in between nSREBP levels and HL mRNA levels:

- transfection of nSREBP1a was found to decrease HL RNA and promoter activity;
- compactin, which increased nSREBP transcriptional activity, presumably through the SREBP1a and 2 isoforms, led to a decrease in HL RNA and promoter activity. Of note, depletion of cholesterol *in vivo* with a bile acid resin and an HMG-CoA reductase inhibitor increased nSREBP2 but decreased nSREBP1 in the liver of mice and hamsters (128;183). However, in McA cells, compactin only slightly decreased mature nSREBP1c levels (117) despite a selective decrease in SREBP1c mRNA and membrane protein due to the inhibition of oxysterol (LXR ligands) synthesis.

- treating cells with sterols, which strongly decreased total nSREBP transcriptional activity (Figure 3-5), is known to lead to a decrease in HL mRNA levels, but to an increase in nSREBP1c activity specifically. Dietary cholesterol in mice led to a marked increase of hepatic SREBP1c expression through activation by oxysterol-activated LXR/RXR heterodimers without affecting SREBP1a or 2. The increase in SREBP1c mRNA resulted in a similar increase in mature nSREBP1c and activation of its lipogenic gene targets (73;126). SREBP1c transcription is induced by cholesterol and repressed by PUFAs. Cholesterol could override the PUFA-induced decrease in SREBP1 mRNA, while PUFA could oppose the maturation of SREBP1c by cholesterol (184). In our experiments, it is likely that PUFAs are absent (defatted medium) or highly limiting (LPDS medium), and as a consequence that nSREBP1c levels would have increased again after 12 hours of treatment (135). Under this hypothesis, the decrease in nSREBP transcriptional activity may have mostly reflected a decrease in nSREBP1a and 2 which are the most active isoforms (121), and may have concealed an increase in total nSREBP mass due to increased nSREBP1c. In this regard, it is of interest that primary hepatocytes as well as McA cells (but not HepG2 cells), have more endogenous SREBP1c than 1a (117;118).

The results presented in this study differ sharply from the results of Busch et al. (74), who found that treating HepG2 cells for 48 hours with mevinolin in LPDS increased HL secreted activity and mRNA by 4.9 and 1.8 fold respectively. Further, whereas a mix of cholesterol and 25-OH-cholesterol was found to inhibit HL expression in our studies, Busch et al. (74) found that 25-OH-cholesterol further increased HL expression when added together with mevinolin. Thus, our studies do not concur with their hypothesis that sterols such as 25-OH-cholesterol regulate HL differentially than HMG CoA reductase. However, in accordance with our studies, statins (compactin or atorvastatin) have been shown to decrease HL secreted activity and mRNA levels in some studies (12;76). During the course of our studies, atorvastatin was found to abolish the oleate-induced

stimulation of HL in cultured hepatoma cells. Oleate resulted in a 50% decrease in nSREBP activity while atorvastatin increased total nSREBP activity by 2.3 fold. nSREBP2 transfection was found to decrease HL promoter activity. Both nSREBP2 transfection and atorvastatin treatment were found to prevent the USF1-induced increase in HL activity of the shortest -305/+13 vector, in a similar way as presented here. The authors suggested that SREBP might mediate the effects of atorvastatin and oleate on HL, possibly through interactions with USF1 (76). Our studies directly confirm these results and further show that: 1) nSREBP-induced downregulation is not isoform dependent since nSREBP1a also decreased HL, 2) the USF1-mediated upregulation, and nSREBP-mediated downregulation of HL promoter activity is effective on a hHL -117/+14 promoter fragment, 3) nSREBP1 and USF1 can interact as shown by co-immunoprecipitation experiments. Together, our results also suggest that nSREBP-mediated down-regulation might act through inhibition of USF1 or other HL activators.

Currently, the few examples of gene downregulation by SREBPs to our knowledge involve either direct binding of SREBP to DNA (146-149), or indirect inhibition through binding to/ antagonizing other transcription factors such as Sp1, HNF4 or LRH1 (150-152). Analysis of the hHL proximal promoter failed to reveal the presence of an SRE. Conversely, several E-boxes are present in the HL promoter, which could potentially bind to either or both nSREBPs or USFs. Because USF is a known activator of HL promoter activity (76;80), this suggested that SREBP could inhibit HL promoter activity by competing with USF for binding to the HL promoter. Here, we found that transfection of USF1 or USF2 could activate human HL promoter activity through the shortest -117/+14 HL promoter construct available (Figure 3-9), and that co-transfection of USF1 with increasing levels of nSREBP1a could reverse this activation in a dose-responsive fashion in rat or human hepatoma cells cultured in LPDS or complete medium (Figure 3-8, Figure 3-9). This nSREBP-induced downregulation was mediated through an element within the -249 to +14 region, and possibly through the shorter -117/+14 sequence.

A dominant negative USF1 construct lacking the DNA binding domain could also activate HL promoter activity in LPDS medium, but not in complete medium. This raises the possibility that the USF activation effect could partly result from the release of a low-level SREBP inhibition of the HL promoter in cells at baseline in LPDS medium.

Using gel-shift assays, we tried to identify factors that could mediate the response to USF and nSREBP transfection. The choice of probes for gel-shift assays was guided by the presence of known DNase1 protected sites and/or the presence of putative binding sequences for known lipid/sterol responsive transcription factors (e.g. USF, nSREBP...).

In gel shifts using nuclear extracts from control, compactin, or sterol-treated cells, we found no specific binding to the -14/+14 and -110/-95 regions of the hHL promoter, despite the fact that those regions covered known protected sites. Therefore, we found no evidence that these regions were involved in sterol regulation. We were able to confirm that HNF1 α could bind to the -70/-48 region of the hHL promoter in supershift assays (Figure 3-11), and found that binding was inhibited by both sterols and compactin treatments. Because binding of HNF1 to this proximal binding site is known to activate HL, inhibition of HNF1 binding could result in inhibition of transcription in both treatments, and this would explain the inhibition of -117/+14 hHL promoter activity in response to compactin treatment (Figure 3-10). This suggests that sterol regulation of the hHL promoter within the -117/+14 region is mediated at least partially through the -66/-52 HNF1 site, whereby sterol treatments could modulate the ability of HNF1 to bind and activate transcription of the HL gene.

HNF4 α was found to bind to the -252/-218 region of the hHL promoter, along with unidentified transcription factors. Again, sterols and compactin treatments inhibited the binding of factors to this region, in particular that of HNF4 α . At the minimum one factor is known to bind this region since the area was covered in DNase1 protection assays. Assuming that binding of HNF4 α and possibly other transcription factors in this region

could further activate HL transcription, inhibition of binding in response to both treatments could potentiate the inhibition of HL transcription.

A single factor, identified as USF1, was found to bind to the –317/-298 region of the hHL promoter (Figure 3-13). Gel shifts using nuclear extracts from control or treated cells showed both sterol and compactin treatments inhibit binding of USF1 to its response element. USF1 is an activator of transcription, and absence of binding of USF1 to its response element would be expected to result in decreased transcription. Of note, this region of the HL promoter was not identified as a protected site in neither of the two existing DNase1 protection studies (59;60).

The –520/-509 region of the hHL promoter covers the –514 HL promoter polymorphism with its proposed functional USF site. The common C-514T mutation disrupts a CACGGG USF functional binding site by lowering the affinity of USF to the element by 4 fold (80). This region was not shifted in gel-shifts using nuclear extracts from either control or treated cells. This does not support the proposed role of USF1 binding in this region to modulate HL activity levels in human populations. Functionally, there is a positive association between the CC patients (with an intact USF binding site) and higher HL activity levels (86). However, the absence of a shift fits in with the fact that this region of the HL promoter was not identified as a protected site in neither of the two existing DNase1 protection studies (59;60).

We identified one factor binding to the –566/-546 region of the hHL promoter as USF1. Binding of factors to the –566/-546, –595/-578 or –929/-906 regions of the hHL promoter was not significantly changed upon sterols or compactin treatment compared to control cells (Figure 3-14, Figure 3-15, Figure 3-16).

Of note, nSREBP1 had putative binding sites within probes 5 and 9, and Sp1 within probe 4, but neither SREBP1, SREBP2, nor Sp1 or Sp3 were found to bind to any of their putative binding sites within the hHL promoter. The absence of direct binding of Sp1,

which commonly interacts with SREBP, further suggests that SREBP regulates the hHL promoter by a mechanism other than DNA binding to a response element.

Without binding directly to the HL promoter, we sought to determine whether SREBP could inhibit HL through binding to known (USF1, HNF1 α) or putative (HNF4 α) HL activators. Binding of SREBP1 to HNF4 α has recently been shown to interfere with the HNF4 α recruitment of peroxisome proliferator-activated receptor- γ coactivator 1 (PGC-1), leading to a decrease in phosphoenolpyruvate carboxykinase (PEPCK) transcription (152). In this case, HNF4 α was shown to bind through its ligand binding/AF2 domain to the transactivation domain of SREBP1. Similarly, direct interaction between SREBP2 and HNF4 was demonstrated by *in vitro* pull-down and *in vivo* co-immunoprecipitation experiments (185). The mechanism for SREBP inhibition of HL could resemble this model, whereby SREBP binding to USF1, HNF1 α and/or HNF4 α would compete for recruitment of a coactivator, leading to a decrease in HL gene transcription. In co-immunoprecipitation experiments using nuclear extracts prepared from control or sterol-treated HepG2 cells, we failed to detect a specific co-immunoprecipitation of SREBP1 with either USF1, HNF1 α , or HNF4 α . We reasoned that this may be due to lack of binding of these factors, or to low sensitivity in our assay where endogenous levels of transcription factors are extremely low. When artificially increasing the levels of transcription factors through transfection of nSREBP1a alone or in combination with USF1, we were able to show that SREBP1a can bind to USF1 and possibly also to HNF1 α and HNF4 α (Figure 3-17). Altogether, this suggests that the SREBP-induced decrease in the transcription of the HL gene could be due to interactions with these or other positive effectors of HL transcription.

These studies provide evidence for a novel aspect of the function of SREBPs in the crosstalk between cholesterol and fatty acid/triglyceride metabolism (Figure 3-18).

Figure 3-18: Proposed model for the regulation of HL by SREBP in the presence or absence of cholesterol

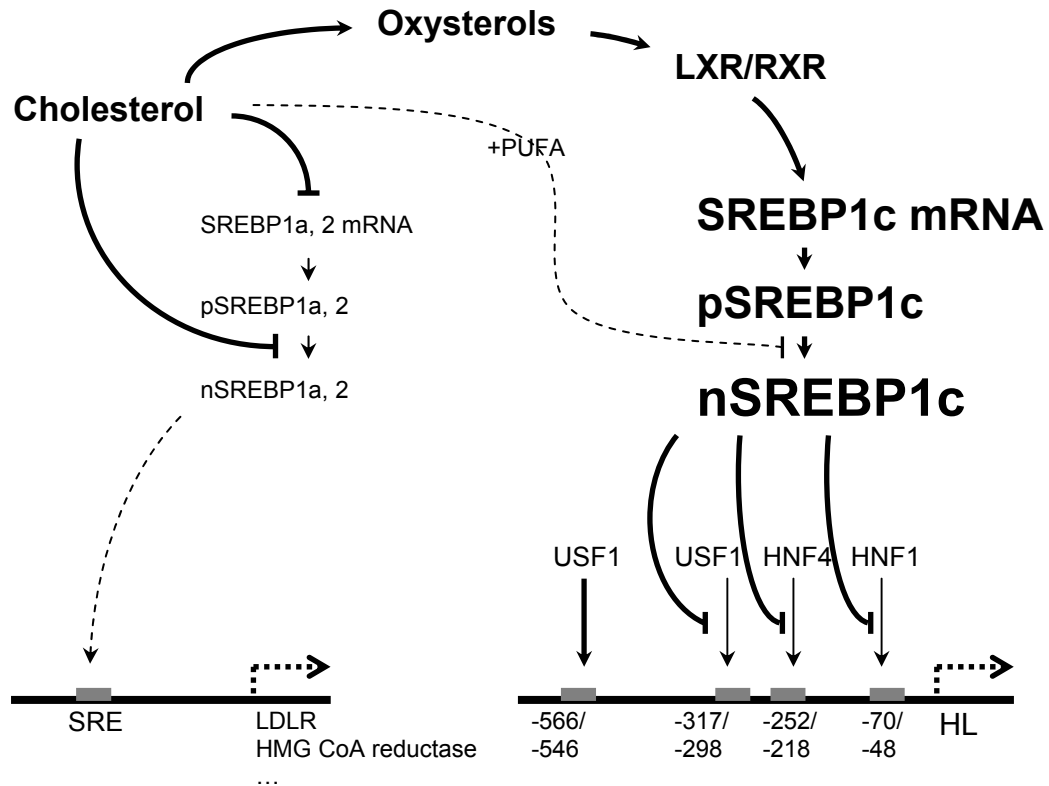
In the presence of cholesterol (upper panel), nuclear SREBP1a and 2 are repressed and sterol responsive element (SRE)-driven genes (shown on the left) are not activated. Oxysterol synthesis is activated, and as a result the LXR is activated by its oxysterol ligands. Expression of the SREBP1c isoform, which requires LXR activation, is increased. The maturation of SREBP1c can proceed in the absence of polyunsaturated fatty acids, resulting in increased levels of nSREBP1c. nSREBP1c has a very low transcriptional activity on an SRE and therefore can not activate SRE-driven genes. However, the high levels of nSREBP1c in the liver can effectively repress HL expression by inhibiting some of its positive regulators (as shown on the right).

In the absence of cholesterol (lower panel), SRE-driven genes (left) are activated by nSREBP1a and 2. SREBP1c transcription is repressed in the absence of LXR activation, and as a result nSREBP1c levels are low. At low levels of total nSREBPs, HL is activated (right).

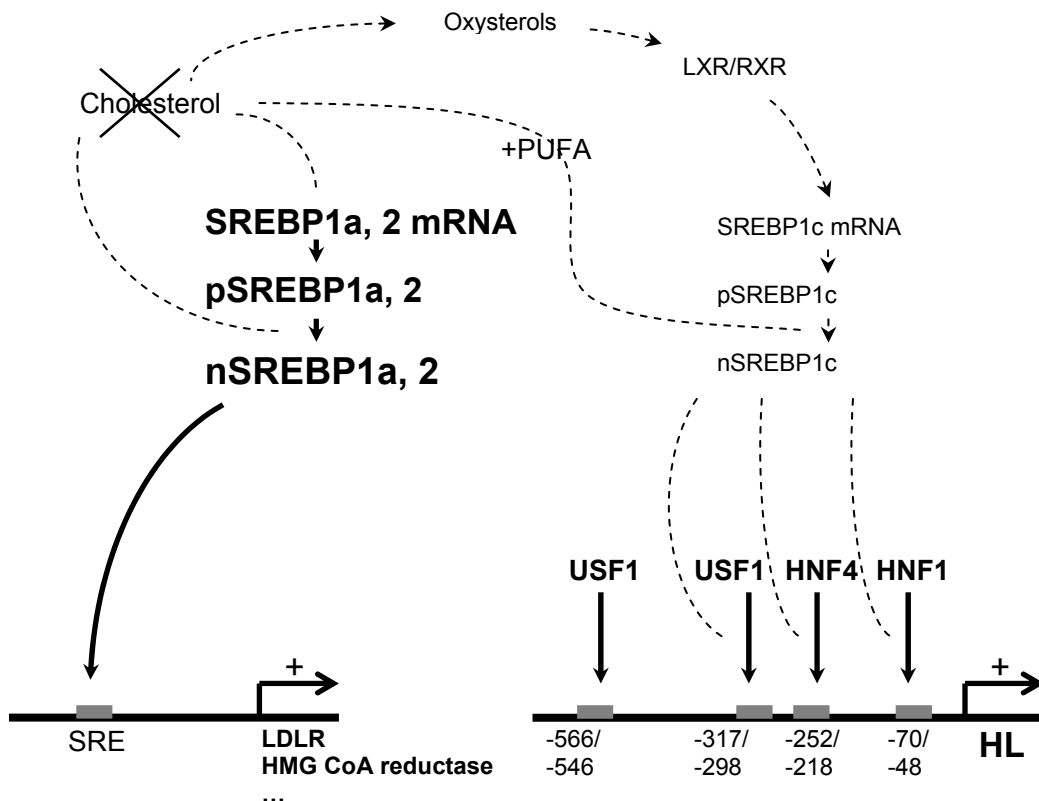
While the proposed mechanisms are different, the end effects of the SREBP-mediated regulation of SRE-driven genes and HL would all be part of a feedback mechanism to maintain stable levels of cholesterol within the cell.

Of note, other genes can be upregulated or downregulated by SREBPs through different mechanisms but are not shown on this simplified diagram.

In the presence of cholesterol:



In the absence of cholesterol:



Chapter 4 - Post-transcriptional regulation of HL by cholesterol

4.1 Introduction

As described in chapter 2, cholesterol decreases the secretion of hepatic lipase by cultured hepatoma cells. Measured secreted rHL protein corresponds to enzyme that has been synthesized and secreted, minus the amount of enzyme that has been degraded post-secretion. Thus, an observed decrease in rHL secretion could be due either to decreased synthesis, increased pre-secretion degradation, decreased secretion, and/or increased post-secretion degradation. All of the assays described above were done in the presence of 100U/mL heparin, a known inhibitor of rHL internalization from the cell surface and subsequent degradation (9), suggesting that post-secretion degradation was not involved in the observed down-regulation of rHL secretion. In this chapter, we further investigated the role of cholesterol on the regulation of HL protein synthesis, protein degradation, and translation efficiency.

4.2 Methods

4.2.1 Reagents

Please refer to chapters 2 and 3 for a list of commonly used reagents as well as tissue culture procedures. Enlightning solution (Dupont NEN- #NEF-974) was used for fluorography to improve detection of ^{35}S since fluor enhances the emission of the label. Cycloheximide (VWR CSPA1002) was prepared fresh as a 5mg/mL stock in water.

4.2.2 Synthesis pulse protocol and immunoprecipitation with protein G for cholesterol experiment

Confluent McA-RH7777 in 100mm collagen-coated dishes (one dish/sample) were preincubated for 4 hours with LPDS McA medium (Cab-O-Sil-treated serum) and then treated for 4 or 24 hours with LPDS McA medium with or without 5mM methyl- β -cyclodextrin complexed at an 8:1 molar ratio with cholesterol. The treatment medium

was aspirated and the cells were rinsed twice with methionine- and cysteine-free DMEM. The cells were then fed 5mL/dish methionine-, cysteine-free DMEM supplemented with 100 (4 hours) or 87.5 μ Ci/mL (24 hours) respectively of Tran-³⁵S-label (70% L-Met, 15% L-Cys, and various non-labeled amino acids, ICN Biomedicals), and incubated at 37C for a 20-minute pulse. During this short period, degradation was assumed to be insignificant. After 20 minutes the medium was aspirated and the cells were rinsed twice with ice-cold PBS before being harvested on ice in RIPA lysis buffer (50mM Tris-HCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.15M NaCl, 10U/mL heparin, 1mM methionine, pH 8.0, supplemented with freshly added protease inhibitors: 1 μ g/mL leupeptin, 1 μ g/mL antipain, 10 μ g/mL benzamidine, 10KIU/mL trasylol from 1000X stock in trasylol solvent, and 1 μ g/mL chymostatin, 1 μ g/mL pepstatin from 1000X stock in DMSO). The lysates were sonicated at 100W for 20 seconds twice, aliquots were removed for TCA-precipitable counts determination (see 4.2.3), and the remaining lysate was centrifuged for 30 minutes at 25,000rpm in a 40.3 rotor at 4C. The supernatant was used to immunoprecipitate rHL using a protocol modified from Doolittle et al.(2). To each supernatant, concentrated BSA was added to a final concentration of 0.1%, and 10 μ g polyclonal anti-rHL antibody was added to cell lysate from a 100mm dish. The immunoprecipitation mixtures were incubated overnight at 4C. 40 μ L or 100 μ L of a 50% suspension of protein G-Sepharose was added to lysate from a 100mm dish starting material and the lysate was incubated for 2 hours. The beads were centrifuged at 8,200g for 5 minutes. The supernatant was discarded and the beads were transferred quantitatively to new tubes, then washed once with RIPA buffer and around 9 times with fresh 0.1% N-lauroyl-sarcosine-sodium salt in PBS. After the last wash, the beads were resuspended in 2X Laemmli buffer and boiled to release the immunoprecipitated rHL. An equal amount of TCA-precipitable counts was loaded and ran on the 10% SDS gel. The gel was fixed in 30% methanol, 10% acetic acid for 1 hour or more, was incubated for 20 minutes in Enlightning for fluorography, and was dried for 2 hours using a slow-

temperature-increase (up to 80C) cycle on a BioRad gel dryer. The intensity of the rHL band was quantitated using a phosphorimager and/or determined by scintillation counting after the band was excised from the gel and dissolved in H₂O₂.

4.2.3 Determination of ³⁵S incorporation in TCA precipitable fraction

This procedure consists of precipitating proteins with trichloro-acetic acid (TCA) as a means to monitor the incorporation of radioactivity into total cellular proteins. It was adapted from Miller and Carrino (186). 5µL/tube aliquots of each cell lysate (after sonication but before centrifugation) or blank lysis buffer were aliquoted into tubes containing 0.9mL of 0.11mg/mL ELISA-grade BSA (used as a carrier protein) and 1.1mM L-methionine. 100% TCA (0.1mL/tube) was added to reach a final TCA concentration of 10%, and the tubes were chilled on ice anywhere from 30 minutes to overnight. Following a 10-15 minutes centrifugation at 13,000 rpm, at 4C, the supernatant was removed and the pellet was redissolved in 75µL 0.1M NaOH. 1mL of 10% TCA was then added for a second round of precipitation, and the mixture was chilled and spun again. The supernatant was carefully removed. A second pulse-spin helped removing any remaining acid that would neutralize the base in the next step. The pellet was then redissolved in 200-300µL 0.1M NaOH (as little as possible to avoid quenching). Following these two precipitations the solubilized protein was quantitatively transferred to scintillation vials filled with 10mL scintillation fluid rinsing the tubes three times with 150µL dH₂O, and counted.

4.2.4 Synthesis experiment and immunoprecipitation with xrHL immunobeads for ACAT inhibitor experiment

In an attempt to improve immunoprecipitation conditions and decrease the amount of bands after immunoprecipitation with an anti-rHL polyclonal antibody, a different protocol was used when the synthesis assay was repeated with an ACAT inhibitor treatment, as described here.

4.2.4.1 Conjugation of xrHL to immunobeads

5mg of anti-rHL polyclonal antibody was used with activated Immunobead matrix (Irvine Scientific) to prepare anti rHL-conjugated immunobeads according to the manufacturer's instructions. Briefly, the antibody was dialyzed overnight at 4C against 0.003M phosphate, pH 6.3 (coupling buffer). The next day, the Immunobead matrix was rehydrated with dH₂O to reach a 10mg/mL suspension in 0.003M phosphate, 0.01%NaN₃, pH 6.3. For conjugation, 10mL matrix was transferred to a 50mL Falcon tube, to which 40mL of coupling buffer was mixed in. The beads were spun down at 1,500g for 10 minutes at 4C, and the supernatant was decanted immediately after the end of the spin. This wash was repeated once by adding a few milliliters of buffer at first, vortexing to resuspend the beads, and then filling the tubes up to maximum capacity. The beads were finally resuspended in around 3mL coupling buffer. The dialyzed antibody was then added to the beads and the volume was adjusted to 10mL with coupling buffer. This mixture was incubated for 1 hour at 4C before 20mg EDAC (1-ethyl-3-[3-dimethylaminopropyl] carbodilimide-HCl) was added and thoroughly mixed in. The mixture was then incubated for 3 hours at 4C. The material was divided into two 50mL Falcons and submitted to a series of washes with ice-cold buffers in a similar fashion as described above, first with PBS, then with 1.4M NaCl-PBS twice, then again with PBS twice. Finally, the beads were resuspended in PBS and incubated for 3.5 hours at 4C to renature the antibody. The beads were then spun down and resuspended in 0.005M phosphate, pH 7.2, pooled, and washed twice with this buffer before being resuspended in 10mL final volume 0.005M phosphate, pH 7.2, 1% BSA. NaN₃ was added to a final concentration of 0.02% for storage at 4C.

4.2.4.2 Synthesis pulse protocol for ACAT inhibitor experiment

The overall procedure was taken from Cisar et al. (21), with a modification in lysis buffer (13). The cell culture medium from 100mm dishes of Fu5AH cells (pools of 2 dishes per sample) was aspirated at timed intervals, and the cells were rinsed twice with

warm methionine- and cysteine-free DMEM (M,C-free DMEM). The cells were then incubated for 10 minutes at 37C, 10% CO₂ in M,C-free DMEM before this medium was aspirated off carefully. 5mL/dish of warm M,C-free DMEM supplemented with 75µCi/mL Tran-³⁵S-label (70% L-Met, 15% L-Cys, and various non-labeled amino acids -ICN Biomedicals) was added and the dishes were placed at 37C in the incubator for 15 minutes. Note that normally under these labeling conditions cell lysate rHL can be immunoprecipitated and detected after a pulse of 5 minutes only. Following the pulse, the medium was aspirated, the dishes were rinsed twice with ice-cold PBS and were placed on ice from now on. Each pool of 2 dishes was harvested with a plastic disposable rubber policeman in 2.5mL (1+1.5mL) lysis buffer (1% Triton X-100, 1% deoxycholate, 10mM Tris-HCl, pH 7.4, supplemented with freshly added 1µg/mL leupeptin, 1µg/mL antipain, 10µg/mL benzamidine, 10KIU/mL trasylol, 1µg/mL chymostatin, 1µg/mL pepstatin, 1µM PMSF). The lysates were transferred to 6mL tubes for the 40.3 Beckman rotor and sonicated at 100W for 20 seconds twice (using a Braun-sonic 1510 probe sonicator equipped with a 4mm diameter microprobe). At this point, 35µl and 5µl aliquots were removed and stored at 4C or -20C for DNA assay or TCA precipitation (see 4.2.3) respectively. Lysates were centrifuged for 30 minutes to one hour at 30,000 rpm, 4C, in the Beckman L8-80 ultracentrifuge (40.3 rotor). The supernatants, including the diffuse fat layers, were transferred to 15mL Falcon tubes for immunoprecipitation.

4.2.4.3 Immunoprecipitation of rHL with immunobeads

rHL from cell extracts was immunoprecipitated using 2 successive incubations with the anti-rHL-conjugated immunobeads (21) as described below. Immediately after the spin following the pulse experiment, 1µg/mL leupeptin, 1µg/mL antipain, 10µg/mL benzamidine, 10KIU/mL trasylol, 1µg/mL chymostatin, 1µg/mL pepstatin from 1000X stocks were added to the cell lysate dilution buffer (1% Triton X-100, 0.15M NaCl, 5mM EDTA, 15U/mL heparin, 50mM Tris-HCl, pH7.5), and the cell extract were diluted with 2 volumes of cell lysate dilution buffer. rHL antibody-conjugated immunobeads were

equilibrated with PBS to eliminate any remnant azide, then 150 μ L of immunobeads was added to each lysate tube, and the immunoprecipitation mixtures were incubated overnight on a rotating shaker at 4C. Tubes were spun at 16,100g for 3 minutes and set on ice. The supernatant was pipeted out. The beads were then transferred to new 1.5mL screw cap tubes (using a P1000 without allowing air bubbles) with 0.8mL buffer A (1M NaCl, 0.2% Triton X-100, 10mM Tris-HCl, pH 7.4). The original tubes were rinsed with 500 μ L wash buffer A to finish washing the tubes and transfer the beads quantitatively. From now on, tubes were placed on ice. Tubes were spun down 3 minutes to pellet, then the beads were washed with 1mL buffer B (0.15M NaCl, 10mM Tris-HCl, pH 7.4), vortexed briefly and incubated with gentle mixing for 5 minutes. For elution, the antibody complexes were released from the beads by heating at 54C in 75 μ L of 2% SDS for 1 hour, and then again in 25 μ L of 2% SDS for 10 minutes. The tubes were spun down each time and the supernatants containing the released lipase were pooled. The beads were then washed with 1mL cell lysate dilution buffer and the supernatants were again combined with that of the SDS releases. Final SDS concentration in the samples was adjusted to ~0.06% (or lower) using cell lysate dilution buffer before a fresh 150 μ L of rHL antibody-conjugated immunobeads was added to each tube. The immunoprecipitation mixtures were incubated overnight at 4C with gentle shaking. The beads were pelleted by centrifugation, the supernatants were aspirated off and the beads were transferred to new tubes in 1.3mL buffer A. The beads were then incubated in buffer A for 1 hour at 4C. Without interruption the beads were successively washed with 1mL buffer A, 1mL buffer C (1M NaCl, 10mM Tris-HCl, pH 7.4), 1mL buffer B, twice with 1mL buffer D (1% Triton X-100, 0.15M NaCl, 1% sodium deoxycholate, 10mM Tris-HCl, pH 7.4), 1mL buffer B, 1mL buffer E (1M NaCl, 0.1% BSA, 10mM Tris-HCl, pH 7.4), and 1mL buffer B. For elution, 85 μ L 2X-Laemmli buffer was added (20% glycerol, 4% w/v SDS, 125mM Tris, 0.0025% w/v bromophenol blue, supplemented with fresh 10% v/v β -mercaptoethanol). The immunoprecipitated rHL was released from the

beads by heating at 95°C for 6 minutes in a heating block and spun down at 10,000rcf for 6 minutes. The supernatants were transferred and a second spin was done to recover all of the supernatant. The entire supernatants were resolved on a 10% polyacrylamide gel under denaturing conditions (1% SDS in the running buffer) using a ^{14}C standard (Sigma CFA756) as a marker. The gel was fixed in 30% methanol, 10% acetic acid for 1 hour or more, incubated for 10-30 minutes in 50% methanol, then for 15-30 minutes in Enlightning solution at room temperature. The gel was then dried on the gel dryer (BIO-RAD Model 583) using a 2 hour slow temperature increase cycle up to 80°C, and exposed to a Cyclone SR screen for 3 hours or to Kodak X-OMAT AR film at -80°C for 6 days.

4.2.5 Preparation of cytoplasmic cell extract

This protocol is adapted from Yukht et al. (187) itself adapted from an original protocol by Walden et al. (188). All buffers were prepared in RNase free conditions. β -mercaptoethanol was always added fresh immediately prior to use. The entire procedure was achieved in one day without delay, at 0-4°C using pre-chilled buffers and equipment. For each condition, seven 100mm dishes of cells were grown and treated as indicated. After treatment, the cells were washed with ice-cold PBS. Cells were scraped in fresh PBS (2mL/dish) and pooled for each treatment in a 15mL graduated conical centrifuge tube. Cells were pelleted by centrifuging for 10 minutes at 3,000rpm, the supernatants were discarded, and the packed cell volume (pvc) was measured using the graduations on the tube. The pvc was resuspended in lysis buffer (50mM Tris-HCl, 35mM KCl, 10mM MgCl_2 , 250mM sucrose, 0.5mM EDTA, pH 7.4, 7mM β -mercaptoethanol) to a final volume of 3 times the original pvc. Cells were allowed to swell on ice for 10 minutes and were homogenized in a homogenizer (type B pestle) with ten up and down strokes on ice. Cells were transferred to eppendorf tubes, and mitochondria and nuclei were pelleted by spinning at 10,000g for 15 minutes. The supernatants were spun at 100,000g (37,000rpm) for 2 hours in the ultracentrifuge (SW65Ti rotor). Solid ammonium sulfate was added to the cytosolic S-100 (high speed supernatant) fractions to 60% saturation

(36.1g ammonium sulfate/ 100mL) and the proteins were precipitated for 30 minutes on ice. Precipitated proteins were collected by centrifuging at 6,000g for 10 minutes in eppendorf tubes and the pellets were redissolved in 300 μ L resuspension buffer (20mM Tris-HCl, 20mM KCl, 10% glycerol, pH 7.4, 7mM β -mercaptoethanol) (189). The cytoplasmic cell extracts were aliquoted in tubes, snap frozen, and stored at -80C. Protein concentration was determined by Lowry after DOC/TCA precipitation, using BSA as a standard (See 3.2.8). Average yield was ~1mg protein/pool of seven dishes.

4.2.6 Preparation of a human HL transcript

The prK5-hHL human HL cDNA expression plasmid, where the hHL cDNA is inserted in between the Not1 and HindIII sites (gift from Dr. Chang SF), was amplified and purified using a Qiagen midiprep, and was finally resuspend in TE (10mM Tris, pH 8.0, 1mM EDTA). Because plasmid purification with this kit yields plasmid contaminated with RNase A, the plasmid was further treated with 200 μ g/mL proteinase K for 30 minutes at 55C in 0.5% SDS, 0.1M NaCl, 10mM Tris, pH 8.0, 1mM EDTA. Subsequently, the plasmid was submitted to one round of phenol/chloroform extraction. Briefly, an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v – from EM) was added to the DNA to be purified, the mix was vortexed vigorously for 10 seconds, and spun down for 2 minutes at top speed at room temperature. The top aqueous phase containing the DNA was transferred to a new tube and extracted with an equal volume of chloroform 24:1 isoamyl alcohol using the same procedure as before. The DNA was then precipitated at -80C for at least 15 minutes with 1/10th volume of RNase-free 5M ammonium acetate and 2 volumes of 100% ice-cold ethanol. The DNA was pelleted by spinning for 5 minutes at top speed, air-dried, and resuspended in RNase-free water at a final concentration of 0.5 to 1mg/mL. The vector was linearized overnight at 37C by digestion with HindIII, and complete linearization was checked on agarose gel. HindIII cut at the very 3' end of the hHL cDNA, right upstream of the plasmid polyA signal, leaving a 5' overhang that does not interfere with phage RNA

polymerases. After linearization, the reaction was terminated by addition of 1/20th volume of 0.5M EDTA, 1/10th volume of 5M ammonium acetate, and 2 volumes of ethanol. The mix was chilled at -80°C for at least 15 minutes, and the precipitated DNA was pelleted by spinning at top speed, 4C, for 15 minutes, before being resuspended in RNase-free H_2O at a concentration of 0.5-1 $\mu\text{g}/\mu\text{L}$. RNA was *in vitro* transcribed from this linearized DNA using the Maxiscript SP6 Kit (Ambion). Sense RNA was generated using SP6 RNA polymerase according to the kit's instructions, extracted with phenol chloroform as described above to get rid of DNase1 and other proteins, resuspended in 40 μL RNase-free water, and stored in 5 μL aliquots at -70°C .

4.2.7 *In vitro* translation experiments

In vitro translation reactions were carried out using the rabbit reticulocyte lysate system, nuclease-treated (Promega L4960) according to the manufacturer's instructions. All reagents were allowed to thaw slowly on ice. The template mRNA was denatured at 65°C for 3 minutes and immediately cooled in an ice-water bath. Redivue™ L- [^{35}S]methionine (1,200Ci/mmol at 10mCi/mL, Amersham Pharmacia, Cat.# AG1094) was used as a source of labeled amino acid. The 50 μL reactions were assembled in 0.5mL polypropylene RNase-free microcentrifuge tubes and the translation reactions were immediately incubated at 30°C for the length of time indicated. Reactions contained 70% Rabbit Reticulocyte Lysate, 20 μM Amino Acid Mixture without methionine, [^{35}S]methionine at 0.4 $\mu\text{Ci}/\mu\text{L}$, RNasin® Ribonuclease Inhibitor (Promega N2111) at 0.8U/ μL , with or without 0.1 μg hHL mRNA or 1 μg luciferase mRNA template, and with or without 0.1 μg cytoplasmic extract (or cytoplasmic extract buffer as a control). The reactions could also be scaled down by half in 25 μL final. After the incubations, aliquots of the reactions (25 μL for hHL reactions, 3 μL for luciferase control reactions) were mixed with 50 μL of 2X Laemmli gel loading buffer, the mixes were boiled for 3 minutes, and the results of the translation reactions were analyzed on a 10% SDS-PAGE. The gel was fixed in 50% methanol, 10% acetic acid for 1 hour or more,

soaked 5 minutes in a mixture of 7% acetic acid, 7% methanol, 1% glycerol to prevent it from cracking during drying, then in Enlightning for 15-30 minutes for fluorography, and was dried for 2 hours using a slow-temperature-increase (up to 80C) cycle on a BioRad gel dryer. The dried gel was subsequently exposed to a Cyclone screen and analyzed by phosphorimaging.

4.3 Results

4.3.1 Decreased rHL protein synthesis upon cholesterol or 58-035 treatment

In order to conclusively show that the observed decrease in rHL secretion could be explained by a decrease in protein synthesis, a 20-minute pulse experiment with labeled sulfur-amino acids was performed. In absolute amount of label incorporation per dish of cells, cholesterol-treated cells incorporated 70.4% ($p<0.05$) and 46.9% ($p<0.02$) of control-incorporated label in hepatic lipase protein at 4 and 24 hours respectively. Relative to total TCA precipitable counts, the ^{35}S label incorporated in hepatic lipase protein was decreased to 74.8% and 57.4% ($p<0.02$) of control cells in cells treated with cholesterol for 4 or 24 hours respectively (Figure 4-1). Statistical analysis was done using Student's 2-tailed unpaired t-test. Thus, rHL protein de novo biosynthesis was decreased in cholesterol-treated cells compared with control cells in LPDS medium.

This result was confirmed using a 24-hour ACAT inhibitor treatment as a means of increasing intracellular free cholesterol concentration at a stable level of total cholesterol. ACAT inhibitor decreased rHL de novo synthesis relative to total precipitable protein to 66% of control (Figure 4-2). Of note, this assay was done using a different immunoprecipitation protocol and the double immunoprecipitation technique did successfully diminish the number of non-specific bands.

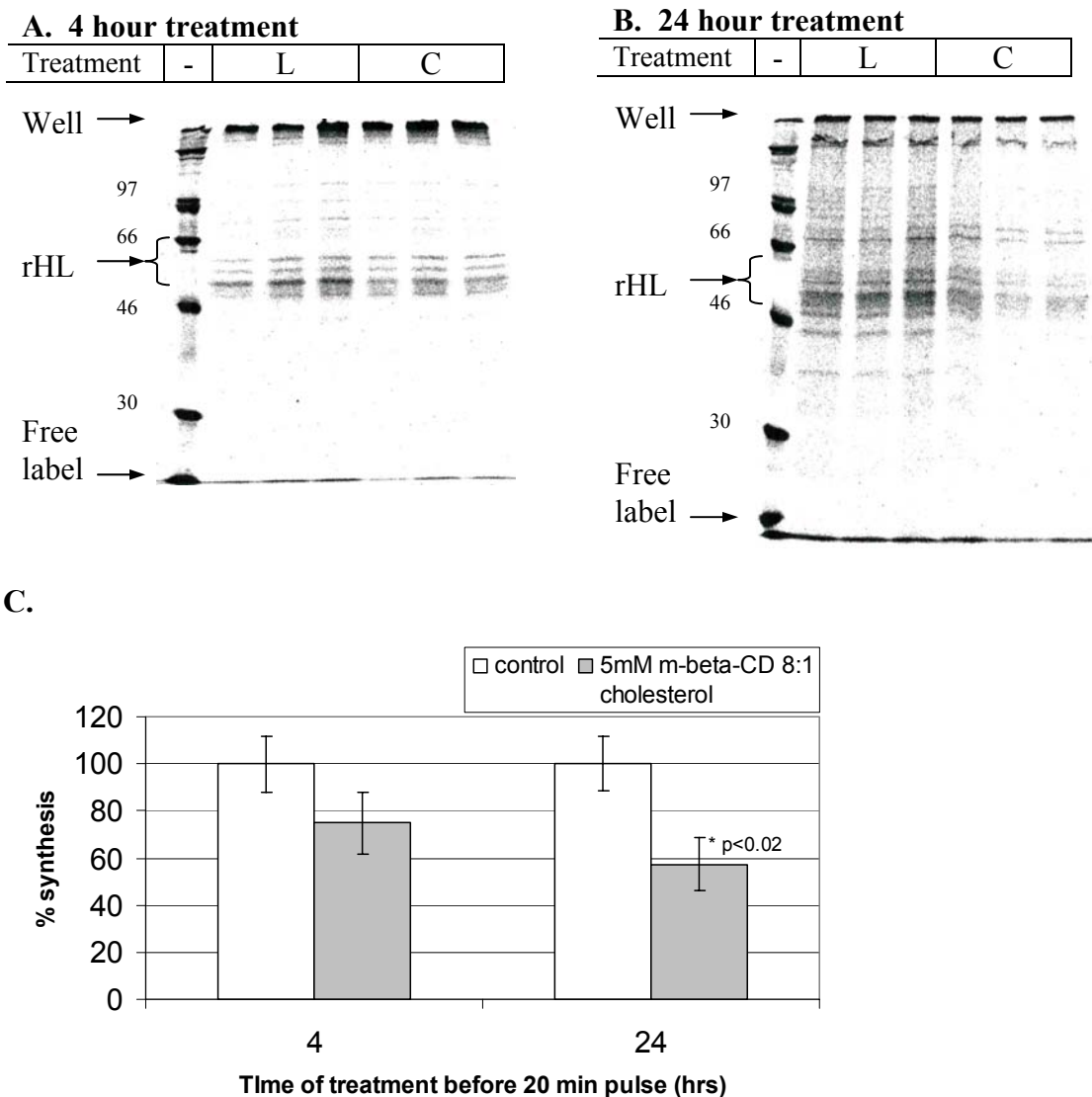
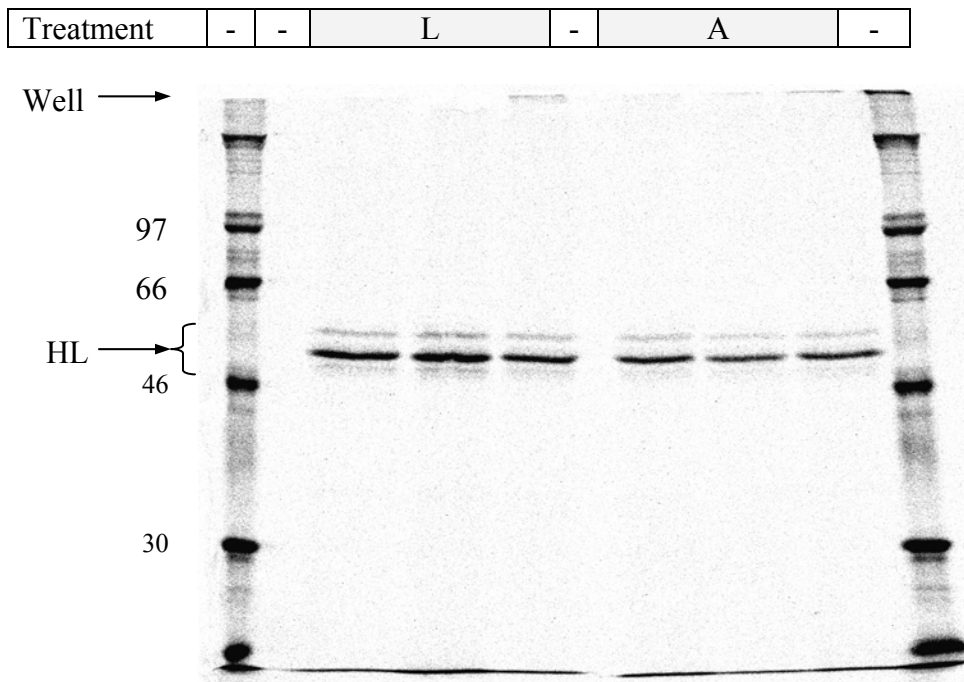


Figure 4-1 rHL protein synthesis is decreased upon cholesterol treatment

McA-RH7777 cells were split into 100mm collagen-coated dishes. At confluency, the cells were maintained in 7mL/dish LPDS McA medium (20% horse LPDS, 5% fetal bovine LPDS prepared by the Cab-O-Sil method, 2mM L-glutamine in MEM, pH 7.4). After four hours the medium was aspirated and replaced by 7mL/dish control LPDS McA medium (L) or LPDS McA medium containing 5mM methyl- β -cyclodextrin complexed at an 8:1 molar ratio with cholesterol (C). After 4 or 24 hours, the media were aspirated and the cells were submitted to a 20 minutes pulse with Tran-³⁵S-label. After the pulse, the lysates were sonicated and aliquots were removed for TCA-precipitable counts determination. The remaining lysates were immunoprecipitated with polyclonal anti-rHL antibody. Equal amounts of TCA-precipitable counts were loaded on 10% SDS-gels for cells treated for 4 hours (Panel A) and 24 hours (Panel B) respectively and the results were quantified (Panel C). Indicated values are relative to control at the respective time-point. Statistical analysis was done using Student's two-tailed unpaired t-test.

A.



B.

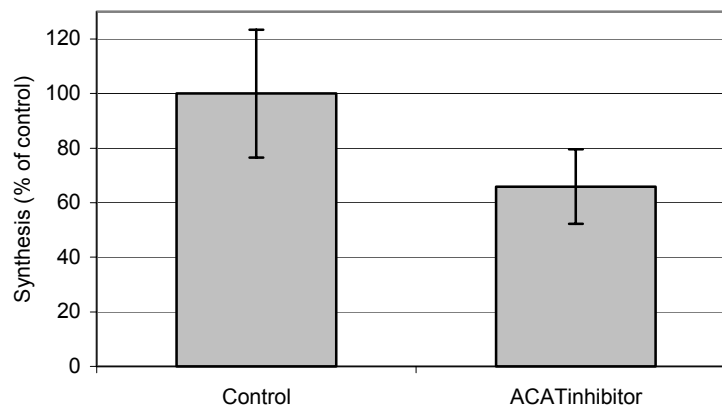


Figure 4-2 rHL protein synthesis is decreased upon 58-035 treatment

Fu5AH cells were treated with LPDS medium (10% LPDS, 2mM L-Glutamine in MEM) with DMSO as a carrier control (control) or 5 μ g/mL 58-035 (ACAT inhibitor). After 24 hours, cells were pulsed for 15 minutes in 75 μ Ci Tran-³⁵S-label /mL methionine- and cysteine-free DMEM. After the pulse, the lysates were sonicated, spun down, and the supernatant was immunoprecipitated with rHL-conjugated Immunobeads. The released lipase was run on a 10% SDS-PAGE and the gel was exposed to a Cyclone SR screen (Panel A). Quantification of total newly synthesized rHL was done by densitometry and hepatic lipase protein synthesis is reported relative to total TCA precipitable counts (Panel B).

4.3.2 rHL protein degradation upon cholesterol treatment

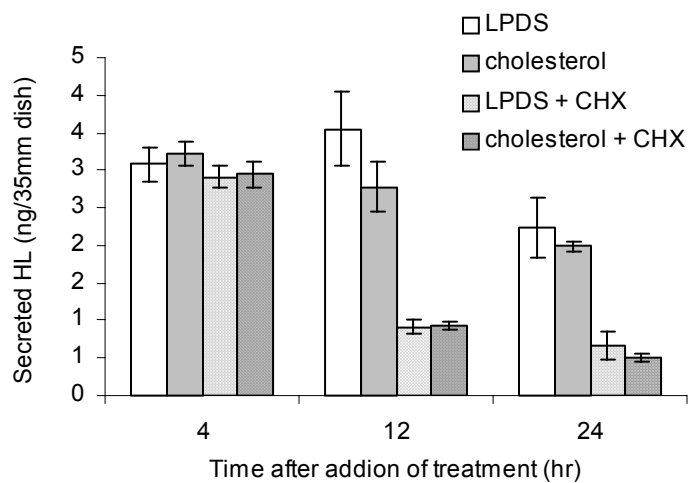
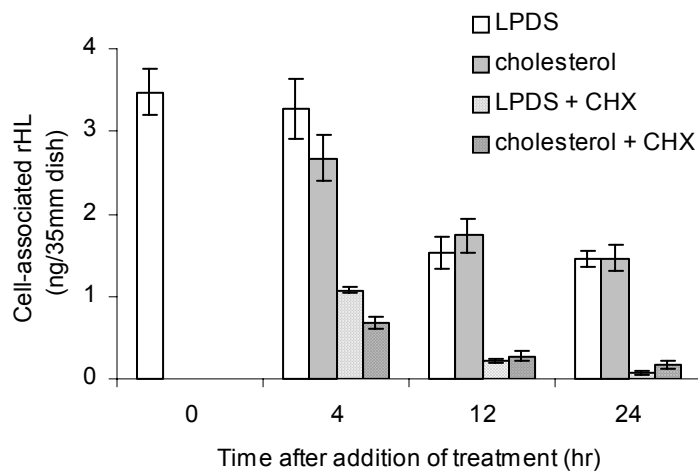
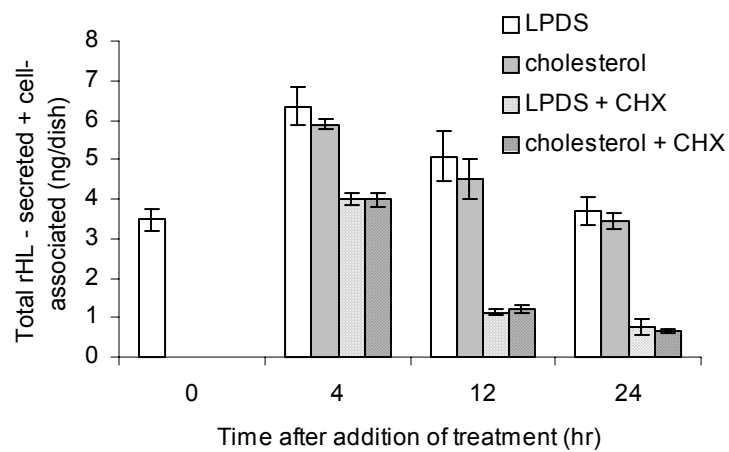
In order to rule out any sterol regulation of HL at the level of internalization and overall protein degradation, the disappearance of rHL in cells and medium upon treatment with cholesterol was monitored in the presence of 5 μ g/mL cycloheximide. Cholesterol was delivered in the form of methyl- β -cyclodextrin complexed to cholesterol (Figure 4-3).

When looking at the sum of cell-associated and secreted HL (Figure 4-3), cholesterol decreased total rHL mass slightly in the absence of cycloheximide. However, in the presence of cycloheximide, there was no difference in total rHL mass upon cholesterol treatment over the course of the experiment, suggesting that protein degradation was not affected. Because the experiment was done in the absence of heparin, this would further suggest that re-internalization of HL from the cell surface was not affected by cholesterol treatment.

These findings were replicated twice in Fu5AH cells using a sterol mixture (cholesterol and 25-OH-cholesterol) or an ACAT inhibitor treatment as a means to increase cell free cholesterol levels (data not shown). Overall, the results suggest that an increase in free cholesterol within the cell does not affect overall rHL protein turnover (internalization and degradation).

Figure 4-3 rHL protein degradation rate is not affected by cholesterol in McA-RH7777 cells

McA-RH7777 cells were split into collagen-coated 35mm dishes. At confluency, two hours before treatment addition, the medium was pre-cleared with Cab-O-Sil-treated LPDS McA medium. At time 0 hour, the cells were treated in LPDS medium without (LPDS) or with 5mM methyl- β -cyclodextrin complexed at an 8:1 molar ratio to cholesterol (cholesterol). When indicated, 5 μ g/mL cycloheximide (CHX) was also included in the original treatment (at 0hr). At the end of the incubation period, medium and cells were collected, and assayed for secreted rHL, or cell-associated rHL respectively. Data is presented either as the sum of cell-associated and secreted HL (top panel) to monitor overall protein degradation rate, or each is presented individually (lower panels). Average \pm SD are shown, with n=3. This experiment is representative of three independent experiments.



4.3.3 Cytoplasmic extract from sterol-treated cells decreases HL translation efficiency in IVT assays

After a four-hour cholesterol treatment, mRNA levels were not altered (see chapter 3) but de novo protein synthesis of rHL was already decreased, suggesting that cholesterol inhibits rHL at the translational level. At a similar level of mRNA, rHL RNA could be subject to editing such as translation efficiency would be decreased. Alternatively, if mRNA was unaltered, rHL translation initiation, elongation, or termination could be altered due to specific binding of a factor to the mRNA. In order to test whether an inhibitory factor was present, we isolated cytoplasmic extracts from cholesterol-treated or control cells and used these extracts in *in vitro* translation reactions using *in vitro* transcribed hHL RNA as a template. A luciferase RNA transcript was also used as a negative control to check for treatment specificity.

Preliminary assays were conducted to optimize assay conditions. First, a titration of hHL mRNA transcript from 0.001 μ g to 0.5 μ g was conducted in a 90-minute reaction. This experiment (data not shown) was used to determine the optimal amount of mRNA substrate for the IVT reaction (0.1 μ g). Second, a time-course of *in vitro* translation reactions was performed with incubation times from 10 to 90 minutes. From this assay (data not shown), it was shown that a 10 minute reaction was enough to observe a product, and that by 20 minutes, the incubation time chosen for subsequent assays, the bands were both intense and within linear range of product formation. After 30 minutes, the rate of product formation decreased and therefore it became impossible to quantify the HL protein synthesis rate upon sterol treatment.

A typical gel for an IVT assay is presented in Figure 4-4. A negative control reaction containing no mRNA allowed for measurement of background incorporation of labeled amino acids, which was not detectable in the range of HL molecular weight. A positive control reaction using luciferase mRNA as a template (Promega) was also included. Finally, three reactions with hHL mRNA as a template were performed, one with no

extract, one with control extract, and one with extract from cells treated for 4 hours with 5mM methyl- β -cyclodextrins complexed at an 8:1 molar ratio to cholesterol. The molecular weight of the hHL translation reaction products was checked by graphing the ratio of the distance from the well to the band to the distance from the well to the dye front as a function of the natural logarithm of the size of the protein molecular weight in Daltons. Using this equation, the size of the observed translated bands was estimated to be at 55.83kDa for the major band, and 49.02 and 46.00kDa for the two minor bands. Thus, the size of the major band was in very good agreement with the expected size of the hHL protein of 55.88kDa as determined by its protein sequence. The two minor bands are most probably hHL synthesis intermediates or hHL degradation products, since in independent synthesis experiments where HL was immunoprecipitated, bands corresponding to the same sizes were also observed (Figure 4-1). From the present experiment, it appeared that extracts from control cells did not affect protein synthesis rate, whereas extracts from cholesterol-treated cells decreased the translation rate of hHL.

The next assay was designed to quantify the inhibitory effect of sterols on HL translation efficiency. Fu5AH cells were treated for 24 hours with a mix of sterols and an ACAT inhibitor, or with LPDS medium as a control before cytoplasmic extracts were isolated. The *in vitro* translation reaction incubation time was set at 20 minutes, within the linear range of HL product formation. Under these conditions, it was found that increasing amounts of extract from sterol-treated cells decreased HL translation efficiency in a dose-dependent fashion, down to 47% of control in the presence of 1 μ g extract (Figure 4-5). In the same experiment, 0.5 μ g extract from control or treated cells did not affect the translation efficiency of a luciferase positive control transcript.

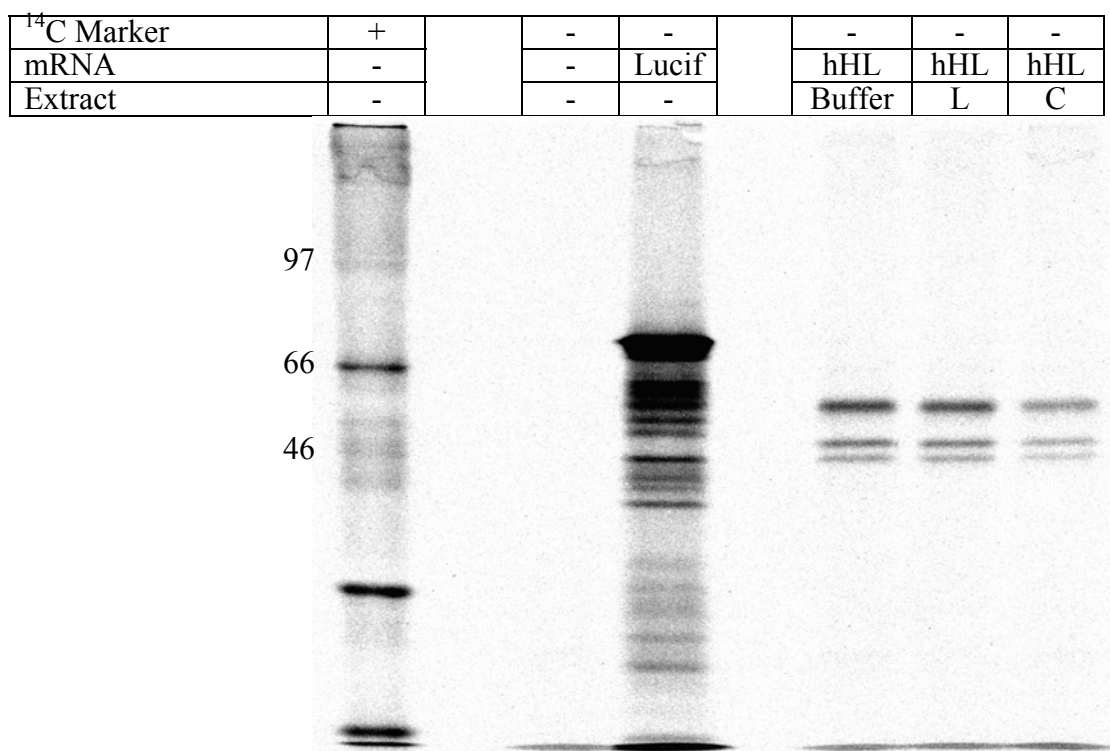


Figure 4-4 Cytoplasmic extract from cholesterol-treated cells decreases hHL translation efficiency

McA-RH7777 cells were split into collagen-coated 100mm dishes. After 24 hours, upon confluency, cells were placed on LPDS McA medium (where the LPDS was prepared by the Cab-O-Sil method) for 4 hours. After this pre-incubation, cells were placed on control LPDS McA medium (L) or LPDS McA medium containing 5mM methyl- β -cyclodextrin complexed at an 8:1 molar ratio with cholesterol (C) for 4 hours. After this treatment, the cells were rinsed with PBS and were immediately used for isolation of cytoplasmic cell extract. *In vitro* translation reactions were carried out using the rabbit reticulocyte lysate system in the presence of L-[³⁵S]methionine, RNasin®, 0.1 μ g hHL or 1 μ g luciferase mRNA, and with or without 0.1 μ g cytoplasmic extract (or cytoplasmic extract buffer as a control). The reactions were immediately incubated at 30°C for 90 minutes and the results of the translation reactions were analyzed on a 10% SDS-PAGE. The dried gel was exposed to a Cyclone screen and analyzed by phosphorimaging.

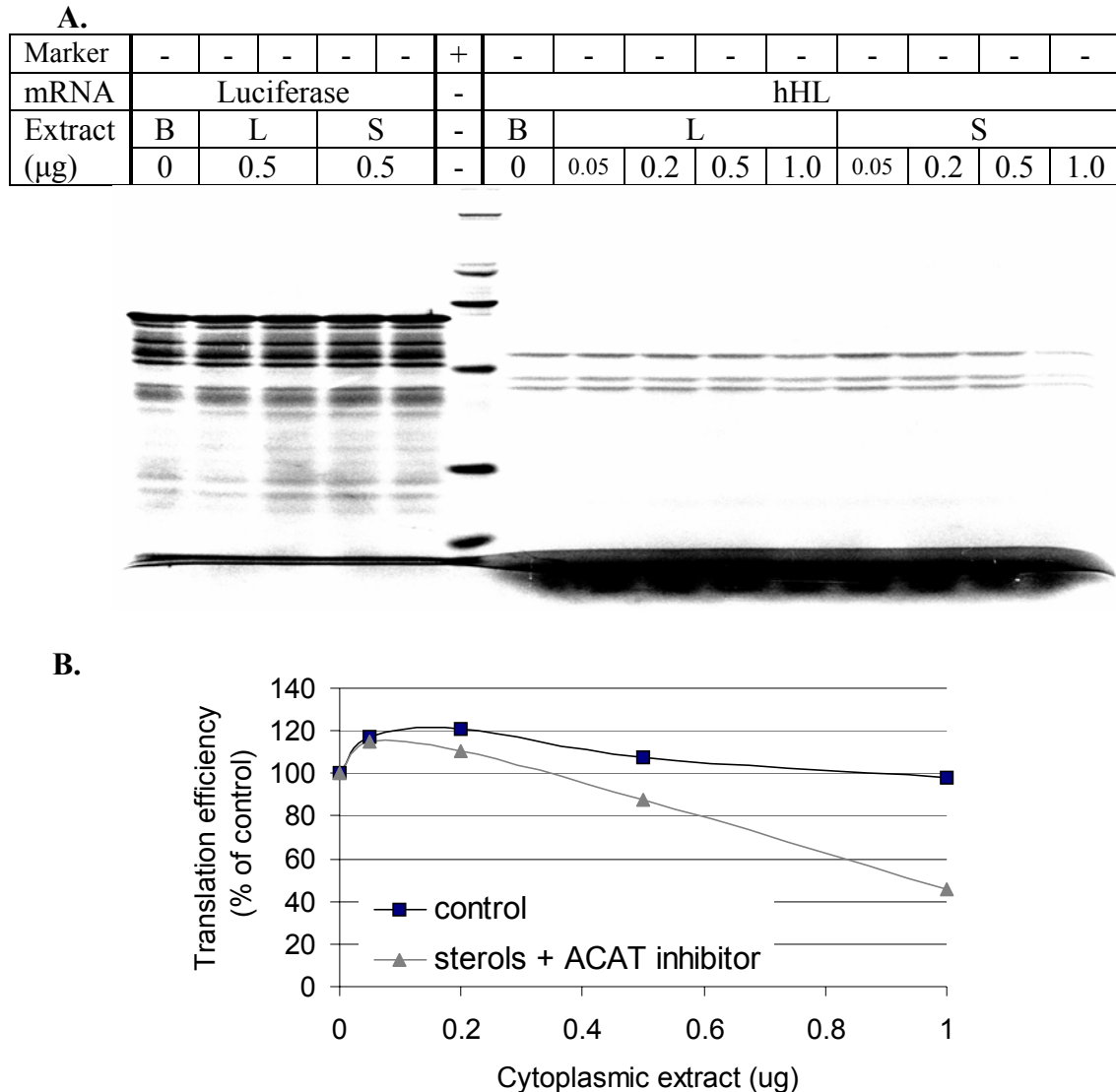


Figure 4-5 Cytoplasmic extract from sterol-treated cells decreases hHL translation efficiency in a dose-dependent manner

Fu5AH cells were split into 100mm dishes. After 24 hours, upon confluency, cells were placed on LPDS medium (10% LPDS prepared by the centrifugation method, 2mM L-glutamine in MEM) as control (L) or LPDS medium supplemented with 10μg/mL cholesterol, 1μg/mL 25-hydroxycholesterol, and 5μg/mL ACAT inhibitor 58-035 (S) for 24 hours. Cytoplasmic cell extracts were isolated immediately after this treatment. *In vitro* translation reactions were carried out using the rabbit reticulocyte lysate system in the presence of L-[³⁵S]methionine, RNasin®, hHL or luciferase mRNA, and increasing amounts of cytoplasmic extract (or cytoplasmic extract buffer (B)). The reactions were immediately incubated at 30°C for 20 minutes and the results of the translation reactions were analyzed on a 10% SDS-PAGE (Panel A). The results of this and an independent experiment were quantified, averaged, and are presented in panel B.

4.4 Discussion

As was discussed in chapters 2 and 3, a four-hour treatment with cholesterol decreased rHL secretion levels at stable mRNA levels. In this chapter, we sought to explain the observed decrease in rHL secretion. A 15-20 minutes pulse experiment in the presence of ^{35}S methionine confirmed that after a four or twenty-four hour treatment with cholesterol, rHL de novo synthesis was decreased. Additionally, treatment with an ACAT inhibitor could also decrease rHL synthesis, suggesting that free cholesterol within the cell, and not total cholesterol, is the trigger for the effect.

Next, we sought to confirm that cholesterol had no effect on HL protein degradation.

As discussed previously, HL secretion experiments were done in the presence of heparin, which prevents HL internalization and subsequent degradation. These experiments suggested that cholesterol downregulation was not due to post-secretion degradation. However, one could not rule out that degradation could occur after protein synthesis but before secretion. In the present experiments, total rHL protein levels (intracellular, cell-associated and secreted protein) were monitored upon cholesterol treatment in the presence of cycloheximide. CHX is a peptidyl transferase inhibitor and therefore it is commonly used as an inhibitor of protein synthesis. In the presence of cycloheximide (and in the absence of heparin), there was no difference in total rHL mass upon cholesterol treatment over the course of the experiment. Overall, these results strongly suggest that HL protein degradation is not affected by cholesterol treatment, whether before or after secretion. While this could be definitively shown by doing a pulse-chase experiment in the presence of labeled methionine, in the absence of any evidence that this mechanism might be involved, this experiment was not conducted.

An interesting side observation could be raised from this experiment by looking separately at cell-associated protein in control cells not treated with cycloheximide. Indeed, cholesterol appeared to have a dual effect on HL cell-associated protein levels. Cholesterol decreased cell-associated HL within the first few hours after treatment, but

this trend reversed after 12-24 hours suggesting the presence of a compensatory mechanism. A rapid effect of sterols is to decrease HL protein synthesis and thus secretion (Figure 4-1). This level of regulation can be fast since translational regulation bypasses the transcription, splicing, and mRNA translocation steps. The delayed compensatory mechanism is unlikely to be due to a change in protein synthesis rate since the decrease in protein translation efficiency is maintained throughout 24 hours of cholesterol treatment (Figure 4-5). An appealing hypothesis is that the secondary increase in cell-associated rHL after a longer cholesterol treatment could be a secondary effect due to a decrease in SREBP levels within the cells, which would release the SREBP inhibition of transcription (see chapter 3) and increase HL transcription. The increased synthesis of HL mRNA template would then slowly compensate for the decrease in the rate of translation efficiency.

We chose to pursue the mechanism whereby cholesterol regulates HL protein synthesis. Indeed, the fact that ^{35}S incorporation decreased at stable mRNA levels (after four hours of cholesterol treatment, see Figure 4-1 and Figure 3-2) strongly indicated that cholesterol inhibits HL at the level of translation.

Translation could be inhibited at the level of translation initiation, which could be determined by doing polysome profile experiments, or at the level of translation elongation and termination. The mechanism could potentially be due to a change in mRNA editing (which could affect RNA stability and/or translation) and/or to the binding of an inhibitory factor to the mRNA.

In order to see if mRNA editing was affected, we attempted to isolate polyA-mRNA from control and treated cells, and perform *in vitro* translation experiments using this polyA-mRNA as a template. The synthesized labeled proteins were then immunoprecipitated with an anti-rHL polyclonal antibody and the immunoprecipitated protein was run on a gel. Unfortunately, the signal from the ^{35}S -labeled rHL band was too weak to draw any reliable conclusion (data not shown).

In order to see if translation efficiency was affected, we performed a series of *in vitro* translation experiments. A common problem of IVT experiments is the presence of RNase contamination in the extract preparations. In the present set of experiments, no difference in translation efficiency was observed when using luciferase mRNA as a template. Because the decrease in translation efficiency was specific to hHL, it is unlikely that it was due to excess RNase in the cytoplasmic extract from cholesterol treated cells, and therefore suggests that the HL mRNA is specifically responsive to some component of the extract from cholesterol-treated cells.

The prK5-hHL vector used for the *in vitro* translation experiments (190) included the full hHL cDNA sequence (NM_000236) with its short 5' untranslated region (about 40 nucleotides), the coding sequence (signal peptide of 66 nucleotides and mature peptide of 1431 nucleotides), and 60 nucleotides 3'-untranslated region. Translation regulation in general is more often mediated by the 5' untranslated region of the mRNA, sometimes by the 3' untranslated region, and less commonly by the coding sequence (191). In general, a 5' untranslated region is thought to have a potential role in translation regulation if it is 100-140 nucleotides or longer and has the potential for establishing a stable secondary structure. The small size of the hHL 5'UTR makes it highly improbable that it contains a regulatory role by providing an Internal Ribosome Entry Sequence. Thus, it seems more probable that HL translation regulation stems from the 3'UTR, or the coding sequence. The regulation of HL at the translational level described in this chapter is not without precedent. Of note, the down-regulation of lipoprotein lipase translation by epinephrine has been shown to be mediated by binding of the catabolic subunit of protein kinase A to the 3'UTR of LPL mRNA (187;192;193). Also, some examples of translational regulation by nutritional factors exist in the literature. For example, vitamin B12 was shown to specifically upregulate the translation of methionine synthase (194). It will remain to be seen if physiological levels of nutrients are effective at regulating HL translation.

Chapter 5 – Future experiments

In rat or human hepatoma cells, cholesterol or the acyl-CoA:cholesterol acyltransferase inhibitor 58-035 markedly decreased secreted HL mass and HL mRNA. Compactin treatment or overexpression of nSREBP1a decreased HL promoter activity and mRNA levels. Additionally, forced expression of nSREBP1a reversed the USF1-mediated activation of hHL promoter constructs. Sterol-responsive binding sites for HNF1, HNF4 and USF1 were identified within the hHL promoter at -70/-48, -252/-218, and -317/-298 respectively. SREBP1a bound to USF1 in co-immunoprecipitation experiments, suggesting that inhibition of HL transcription by cholesterol or compactin may occur through SREBP1 interaction with USF1. These experiments have provided evidence for a novel aspect of the function of SREBPs. The next step will be to define the molecular mechanism responsible for the inhibitory action of nSREBP. SREBP may bind directly to USF1, thereby inhibiting the binding of USF to the HL promoter and/or to its co-activators, and inhibiting initiation of transcription.

It is now clear that transcriptional activity is not the best indicator of nuclear SREBP levels in terms of HL inhibition. A precise assessment of the levels of each functional isoform upon sterol or compactin treatment would be extremely useful to more precisely delineate the regulation of HL transcription. The use of currently available antibodies against SREBP1 (non-specific for isoforms 1a and 1c) and 2 in immunoblots could already provide an estimate of the levels of SREBP1 and 2 within membranes or nuclear extracts. It may be productive to investigate the possibility of producing peptide antibodies specific for SREBP 1a and 1c.

For practical reasons all the studies reported in this thesis employed liver cell lines. It is well established that the proportions of the various SREBP isoforms in these cell lines does not reflect their concentration in liver cells. In particular, SREBP 1c levels are in

general lower in liver cell lines than in primary liver cells. Future work on regulation of HL should be carried out in primary liver cells.

HNF1 is a known activator of HL. Our studies have identified an HNF4 binding site within the human HL promoter. A functional role of HNF4 in regulating HL could be assessed in transactivation assays. Both HNF1 and HNF4 could potentially interact with SREBP, as has previously been shown in the case of HNF4 (152). Co-immunoprecipitation experiments in HNF1 or HNF4 and SREBP transfected cells could determine whether SREBP could decrease binding of these factors to their binding elements within the HL promoter in a similar fashion to USF1.

It would be essential to show that these *in vitro* experiments have physiological significance within cells. Chromatin immunoprecipitation experiments could be used to show direct binding of USF1, HNF1, and HNF4 to the HL promoter and to monitor an expected decrease in the binding of these factors upon inhibition by sterol or compactin treatment, or nSREBP transfection.

Post-transcriptionally in rat cells, cholesterol or 58-035 decreased rHL protein synthesis, at least partly through a decrease in HL translation efficiency. The decrease in translation efficiency in *in vitro* translation assays suggested the presence of an inhibitory factor in the cytoplasmic extracts from sterol-treated cells. In order to conclusively show that an inhibitory factor binds to the HL mRNA transcript or alternatively that an activator is released, an mRNA gel shift experiment could be performed. Assuming a shift was found, this gel shift experiment could also be used to refine the location of protein binding on the RNA by using probes spanning different regions of the mRNA. The functionality of the identified region of the mRNA could later be confirmed by using the sense mRNA as a competitor for binding to the binding protein in *in vitro* translation assays, or by cloning it into a vector to drive the translation of a heterologous reporter gene. The identity of the RNA-binding protein could be probed in supershift

experiments. Alternatively, after partial purification of the extract containing the inhibitory activity the protein could be identified by a proteomic approach, using tryptic digestion, followed by liquid chromatography-mass spectroscopy.

Appendix A- Lipoprotein-deficient serum (LPDS) preparation by density gradient centrifugation method

This protocol is adapted from Busch et al., 1990, JBC, 265(36): 22474-9.

1. Thaw required amount of FBS
2. Make 1.006g/mL solution
Add 11.4g NaCl in 1L volumetric cylinder
Fill with dH₂O to 1L
3. Make ~1.478g/mL solution
Add 78.32g NaBr to 100mL of (1.006g/L solution) (this will yield a volume superior to 100mL) and heat to dissolve if necessary
Check density of new solution by measuring the mass of 10mL (use volumetric pipette in 344 drawer under luminometer)
Record exact density d_D : _____ g/mL

4. Adjust FBS to a final density of 1.21g/mL

$$V_D = V_s(d_f - d_i) / (d_D - d_f) = \underline{\hspace{2cm}}$$

Where V_D = volume of diluent to be added to V_s
 V_s = initial sample volume
 d_f = desired density (1.21g/mL)
 d_i = initial density (1.006g/mL)
 d_D = diluent density

Reference: Havel RJ et al, J Clin Invest, 34:1345 (1955).

Mix V_s (FBS) with V_D (diluent solution at d_D) to reach a final volume of _____ (note 330mL total needed to fill all 8 tubes in rotor).

5. Fill Quick-Seal tubes (Beckman #342414 – 1x3½in) up to the very top (~40mL) leaving air only in half of the stem (air bubbles in the tubes may cause the tubes to collapse during centrifugation). Using a syringe with a metal adaptor, very fine polyethylene tubing can be used and filling can be achieved from the bottle of the tube.

Note: if needed a solution of density 1.21 can be obtained by mixing 10mL of solution d1.006 with 7.61mL of solution d1.478.

Add metal top, and use the machine to heat up the metal, which will transfer the heat to the stem and melt the plastic such as it seals. The stem should almost disappear as you press down.

Remove metal top and apply gentle pressure to check for good sealing.

Rinse the exterior of the tubes with water to prevent damage to the coating of the titanium rotor.

Add the red metal caps and place the tubes in the 60T1 rotor.

6. Spin for >40hours (on hold setting- no specification of time) at 55,000rpm, at 15C (not too cold!) in the Beckman ultracentrifuge.

7. Make buffer for dialysis:

To make 10mM K phosphate final at pH 7.4:

	Final molarity	1L	6L
K phosphate monobasic (MW 136.09)	1.9mM	0.26g	1.55g
K phosphate dibasic (MW 174.18)	8.1mM	1.411g	8.465g
NaCl	120mM	7.0128g	42.08g
KCl	2.7mM	0.201g	1.21g

Check that pH is 7.4

Store at 4C

8. Stop the centrifuge –inactivate the brake.

Record total # of rotations: _____

Remove tubes gently with a pair of hemostats.

9. Make sure tube cutter is greased.

Set tube cutter to cut the tube at 5.1cm from the bottom of the tube.

Cut tube, and leave blade in.

With a 16 gauge needle, make 2 holes in top of tubes and empty the top compartment. Remove the top part of the tube and empty the last bit of fluid in the upper compartment with a Kimwipe.

Remove the blade.

Using a plastic transfer pipette, transfer the content of the lower compartment to a glass cylinder on ice. Make sure to rinse the sides of the tubes (either with delipidated plasma or with the PBS-K dialysis buffer) to resuspend any aggregated protein and recover it.

Recover all tubes in a total volume slightly below that of the original volume of serum used i.e. _____.

10. Dialyze ON against 2L PBS-K dialysis buffer at 4C.

Change of buffer in the morning and again after a few hours.

11. Recover dialyzed serum, record total volume: _____ and filter-sterilize in hood for future tissue culture use. Note that total volume should be same as starting FBS volume. If not, adjust with PBS-K.

12. Optional: check that osmolarity of solution is around 300mosm.

Note: to separate HDL, first get rid of less dense lipoproteins, then use solvent density of 1.21 and spin at 40,000rpm, 40hours, no brake, at 17C.

Reference: Hatch FT and Lees RS, Adv Lipid Res, 6:33 (1968).

Appendix B - Preparation of lipoprotein, fatty acid-deficient serum

A. Lipoprotein-deficient serum (LPDS) preparation using the cab-O-sil method.

This protocol was adapted from S R Behr's PhD thesis, 1983. It removes all lipoproteins from the plasma (Weinstein DB, A single-step adsorption method for removal of lipoproteins and preparation of cholesterol-free serum, *Circulation* **59** and **60**, Suppl. II, Abstract 204, 1979).

1. Thaw required amount of FBS
2. Add 2% w/v Cab-O-sil (this is 2g/100mL) to the FBS
3. Incubate at 4C for 4hours with occasional gentle shaking
4. Spin for 30 min at 20,000rpm using a Sorvall SS-34 rotor (can take 8 tubes with ~35mL each i.e. total of 280mL at one time)
5. Filter-sterilize supernatant in tissue culture hood

B. Proceed with an isopropyl ether/n-butanol extraction to eliminate remaining free fatty acids

This protocol was used by Hannah et al., *JBC*, **276**(6):4365-72, 2001, and was based on an original protocol by Cham and Knowles, *JLR*, **17**:176-81, 1976.

Constituents needed:

- ✓ 800mL isopropyl ether
- ✓ 400mL n-butanol (also called 1-butanol)
- ✓ 500mL FCS

Procedure:

1. Thaw 500 mL FCS
2. In hood, pour the FCS in a 2L flask
3. Add 600mL isopropyl ether and 400 mL of n-butanol (measure solvents in glass cylinder)
4. Incubate in hood 20 minutes at RT, then 20 minutes on ice – swirl regularly to ensure good mixing of the aqueous and organic phases
5. Centrifuge at low speed (≤ 2000 rpm for a few minutes, original procedure called for 2000rpm, 2 minutes). (Note: Can use 50mL Pyrex tubes and spin them in IEC centrifuge using the 921 rotor, 6 tubes at a time, do not forget rubber cushions at the bottom of the gaskets, balance carefully, spin for 10 minutes at 1000rpm). After spinning, there are 3 phases, an upper organic phase, a middle foamy phase, and a lower water-soluble phase.
6. Aspirate organic phase in aspirator flask, as well as middle phase, which should be minimized as much as possible (by spinning harder).
7. Pool all lower phases together in a flask.
8. Repeat steps 5 to 7 as needed.

9. Add 200mL isopropyl ether to the lower phases mixture. This step is to ensure that there is no residual n-butanol in the water-soluble phase.
10. Repeat steps 4 to 8.
11. Flush with a stream of N₂ for 2 hours. Evaporation under nitrogen gas can be achieved with the tubing above or inside the mixture. There should be around 500mL after this step.
12. Freeze-dry.
 - a. Use the speedvac but bypass the concentrator. Unscrew the tubing behind the concentrator and tighten it on the slightly greased glass adaptor (Stover lab) that fits the large vacuum flask (holds 500mL easily – also from Stover lab).
 - b. Shell-freeze the defatted serum. Pour the serum in the vacuum flask, add a little grease on the outside of the flask so it seals well with the rubber cap, “close”, and place the flask in an ethanol and dry ice bath. Swirl so that it freezes in layers, with the maximal surface possible. Note that it is worth doing this in several times if the starting volume is 500mL.
 - c. Once frozen, connect the rubber cap to the greased glass tubing. Leave in a dry ice bath or else the liquid will thaw on the edges, the whole mass will be sucked up. Apply the vacuum.
13. Resuspend in 200mL distilled water.
14. Dialyze against 6L PBS (10mM Pi).
15. Adjust final volume to 500mL with PBS.
16. Spin at 10,000rpm, 30 minutes in SS-34 rotor (Sorvall) to pellet insoluble protein and filter-sterilize the supernatant.
17. Store in multiple aliquots at –20C.

NOTES:

- ✓ this procedure does not affect ionic strength or pH.
- ✓ In Hannah paper:

Mean Free Fatty Acids: 840μM before procedure, 4.4μM after

Mean Cholesterol: 280μg/mL before, 7.5μg/mL after

Mean Triglycerides: 600 μg/mL before, 23 μg/mL after

Appendix C - Preparation of methyl beta cyclodextrin solutions

This protocol was derived from the protocol by Christian et al., Use of cyclodextrins for manipulating cellular cholesterol content, *J. Lipid Res.*, **38**: 2264-72, 1997.

Methyl beta cyclodextrin (Sigma C-4555, MW 1317-59, average: 1338)

Notes:

- Store solid tightly sealed at RT.
- Need 34mg methyl beta CD to make 10mL of 2.5mM.
- Solutions can be stored several months at 4C. Over a 6-day period, there is no loss in the CD: cholesterol complexes if stored at RT in glass containers, but there is a 50% loss of cholesterol if stored in plastic containers.
- Do not add aqueous solutions to the cholesterol-CD complexes but dilute with stock CD solutions if complexes with less cholesterol are desired, otherwise cholesterol can precipitate out

To make 10mM Methyl beta cyclodextrin solution:

- 10mM is 13.38g/1000mL MEM
i.e. _____g/_____mL MEM
- Stir at RT for 30 minutes
- Filter-sterilize through 0.2um
- Store at 4C up to several months in glass container

To make 2mM or 5mM Methyl beta cyclodextrin medium:

	2mM Me beta CD final	5mM Me beta CD final
10mM Me beta CD in MEM	20 mL	50 mL
Cab-o-sil treated FBS (5% final)	5 mL	5 mL
Cab-o-sil treated horse serum (20% final)	20 mL	20 mL
L-glutamine (2mM final)	1 mL	1 mL
MEM	54 mL	24 mL
	100 mL	100 mL

Check that pH=7.4 with pH paper (and adjust if necessary, usually not required)

To make 50mg/mL stock solution of cholesterol in 1:1 chloroform:methanol

50mg/mL (MW: 386.67) is 129.3mM

- In glass scintillation vial with Teflon lining: 500mg/10mL
i.e. _____mg/_____mL
- Flush vial with N₂ after each use.

To make 5mM Methyl beta CA 8:1 cholesterol molar ratio medium:

Note that the theoretical final concentration of cholesterol would then be 625uM, in practice though, one does not know what the final concentration is b/c the solution is saturated in cholesterol and the excess is filtered out.

- $129.3/0.625=206.9$ dilution factor
- Final volume needed _____ mL/ $206.9 =$ _____ mL of 50 mg/mL stock solution of cholesterol to use in next step
- Dry the cholesterol from previous step under N_2 in a glass ampoule of adequate volume capacity – try to get the cholesterol well dispersed on the surface
- Add the final volume of 5mM Methyl beta CD medium to the dried cholesterol
- Vortex to help resuspension
- Sonicate with tissue culture sonicator in waterbath for 3 minutes at 37C
- Rotate overnight in 37C waterbath under constant low flow of N_2
- Adjust pH to 7.4 (using pH paper strips)
- Filter-sterilize through 0.2 um filter with prefilter

Appendix D - Rat Hepatic Lipase enzyme-linked immunosorbent assay (rHL ELISA)

This protocol is adapted from Cisar and Bensadoun (Enzyme-linked immunosorbent assay for rat hepatic triglyceride lipase, *J Lipid Res.*, 26(3): 380-6, 1985).

Notes: this assay does not cross-react with human hepatic lipase (hHL), chicken lipoprotein lipase (cLPL), or human LPL (hLPL).

✓ **0.1M Carbonate-Bicarbonate Coating buffer (pH 9.6):**

Na ₂ CO ₃	1.59g
NaHCO ₃	2.93g
NaN ₃	0.2g
dH ₂ O	fill to 1L

✓ **PBS-Tween (pH 7.4)**

0.2M NaH ₂ PO ₄ stock	140 mL
0.2M Na ₂ HPO ₄ stock	660 mL
NaCl	140g
0.05% tween	8g
dH ₂ O	fill to 16L

Add all compounds into 4 liters and pour into Nalgene Lowboy dispenser, then add 12L water and check pH.

✓ **1% BSA PBS-Tween**

10g BSA into 1L PBS-tween
Filter solution through Whatman #1 filter

✓ **Substrate buffer (make fresh):**

0.1M citric acid stock	12.15mL
0.2M Na ₂ HPO ₄ stock	12.85mL
ddH ₂ O	25.0 mL (pH~5)

Just before use add in this order:

0-phenylenediamine (OPD)	20mg (carcinogenic)
30% H ₂ O ₂	20μL

✓ **2.5M H₂SO₄**

18M H ₂ SO ₄ stock	69.4mL
dH ₂ O	430.6mL

Start with ~400mL water, add acid slowly and swirl (exothermic reaction). Let cool then bring up to 500mL.

✓ **4X rHL sample buffer (pH 7.4)**

Note: 1X buffer is 1M NaCl, 1% BSA, 10mM Pi, 0.05% tween 20, pH 7.4.

NaCl	117g
BSA (ELISA grade)	20g
Tween 20	1.0g
0.2M Na ₂ HPO ₄ stock	82.5mL
0.2M NaH ₂ PO ₄ stock	17.5mL
dH ₂ O	Fill to 500mL

Filter- sterilize (0.2µm filter)
Dilute 1:4 in dH₂O to obtain 1X rHL sample buffer.

Procedure:

Each of the following incubation steps may be done either for (2 hrs @ 37C), or overnight @ 4C in which case better sensitivity is achieved.

Note: (96 wells/plate) x (200µL/well) = 19.2mL/plate (always make up extra)

1. Coating

- ✓ Dilute goat polyclonal anti-rHL in carb-bicarb coating buffer to reach a final concentration of 1µg xrHL/200µL (ie 1µg/well, also equivalent to 5µg/mL)
 - ✓ Add 200µL/well
 - ✓ Incubate ON (or up to a week) at 4C or (2hrs, 37C)
- It is not a good idea to try to use old plates. At best coat plates the day before.

2. Blocking with 1% BSA PBS-tween

- ✓ Wash plates 3x with PBS-tween using the ELISA plate washer
- ✓ Add 300µL/well (ie 2x 150µL) of 1% BSA PBS-tween
- ✓ Incubate (2hrs, 37C) or (ON, 4C)

3. Sample addition

a) Make rHL Standard Curve:

- ✓ 1 tube contains 10µL of 3.5 ng/µL rHL-1/16/97 (-80° freezer)
- ✓ Add 690 µL 1X sample buffer --> 700 µL 1:70 dilution (10 ng/200µL)

ng rHL per well	1X sample buffer(µL)	1:70 standard (µL)
0.00	1000	0
0.02	998	2
0.05	995	5
0.10	990	10
0.15	985	15
0.20	980	20
0.30	970	30
0.40	960	40
0.55	945	55
0.70	930	70
0.85	915	85
1.00	900	100

This will give a range from 0-1ng rHL/well (delivered in 200 μ L). Use quadratic regression fit for analysis.

- b) Dilute samples to be assayed in 1X sample buffer to deliver approximately 0.5ng/well (i.e. concentration of 2.5ng/mL)
- c) Wash plate 3x with PBS-tween using the ELISA plate washer (if no PBS-tween is added to the wells until loading, make sure to load fast such as the wells do not dry out)
- d) Add samples to the plate (200 μ L/well)
- e) Incubate ON @ 4 for better sensitivity (if one step needs to be done ON, this is the one) or else 2 hours at 37C.

4. Conjugate

- ✓ Dilute (goat polyclonal) xrHL-HRP conjugate in 1X sample buffer (depending on conjugate batch, usually 1:5000 to 1:20000 dilutions are appropriate) – see preparation of HRP conjugate below.
- ✓ Wash plates 3x with PBS-tween using the ELISA plate washer
- ✓ Add 200 μ L/well of diluted conjugate
- ✓ Incubate (2hrs, 37C) or (ON, 4C)

5. Revelation

- ✓ Mix buffer for substrate, add OPD and then H₂O₂ (as described above)
- ✓ Wash plates 6x with PBS-tween using the ELISA plate washer
- ✓ Add 200 μ L substrate buffer/well at timed intervals from top to bottom
- ✓ Leave plate in the dark (usually ~30minutes) to allow color development
- ✓ Add 50 μ L H₂SO₄/well to stop the color changing reactions, use the same time intervals from top to bottom, and record the exact length of revelation
- ✓ Read absorbance of each well at 490nm.

Appendix E - Gluteraldehyde conjugation of horseradish peroxidase (HRP) to immunoglobulin G (IgG)

Adapted from The Enzyme Linked Immunosorbent assay: a Guide with Abstracts of Microplate Application, by Voller A, Bidwell DE, Bartlett A, Dynatech Europe, Borough House, Rue du Pre, Guersney, Great Britain, p. 42-50.

Solutions:

- ✓ 0.15M NaCl
 - 35.06g NaCl in 4L dH₂O

- ✓ PBS (10mM phosphate-buffered saline), pH 7.4
 - NaCl: 35.06g
 - NaH₂PO₄: 1.05g
 - Na₂HPO₄: 8.68g
 - Adjust volume to 4L with dH₂O

- ✓ Buffer for HRP solubilization, pH 6.9
 - NaCl: 877mg (final 0.15M)
 - NaH₂PO₄: 621mg (or 10mL of 0.2M stock) (0.1M phosphate final)
 - Na₂HPO₄: 1.47g (or 40mL of 0.2M stock) (0.1M phosphate final)
 - Add 5mL of gluteraldehyde 25%
 - Adjust volume to 100mL with dH₂O, adjust pH

- ✓ 1M Carb-Bicarb buffer, pH 9.5
 - Na₂CO₃ (MW 105.99): 3.18g (i.e. ~0.3M)
 - NaHCO₃ (MW 84.01): 5.86g (i.e. ~0.7M)
 - Adjust volume to 100mL with dH₂O

- ✓ 0.2M lysine
 - 3.65mg lysine in 100μL H₂O

- ✓ Saturated (3.9M) ammonium sulfate, 10mM Pi, pH 7.0
 - Ammonium sulfate: 51.48g
 - NaH₂PO₄: 94mg
 - Na₂HPO₄: 84mg
 - Dissolve in <100mL dH₂O, adjust pH to ~7.5 with NaOH pellets, then to 7.0 with HCl
 - Adjust final volume to 100mL with dH₂O
 - Filter through Whatman #1 paper

Day 1:

1. Preparation of IgG: use 5mg total, ideally at a final concentration of 5mg/mL in 0.15M NaCl (a more dilute source of IgG is OK, further in the procedure, volumes will need to be adjusted to reach the same final concentrations). Dialyze sample overnight in 2 liters 0.15M NaCl at 4C.
2. Solubilization of HRP: dissolve 10mg HRP in 200 μ L 0.1M phosphate, 0.15M NaCl, 1.25% v/v glutaraldehyde, pH 6.9, (use 11mg in 220 μ L to be safe). Incubate overnight at room temperature.

Day 2:

1. Pass solubilized HRP down Sephadex G-25M column (PD-10, Pharmacia):
 - a. Rinse empty column with ~60mL 0.15M NaCl
 - b. Allow liquid to run down to column level
 - c. Add HRP solution in thin layer
 - d. Wash through with saline (have a tube ready, as HRP goes down quickly)
 - e. Collect colored HRP into test tube
 - f. Bring HRP sample volume to 1mL (or 1.1mL) with saline
2. Mix IgG and HRP:
 - a. Mix 1mL (or total volume if >1mL) IgG with 1mL (1.1mL) HRP in a 15mL Falcon tube
 - b. Add 100 μ L of 1M carb-bicarb buffer, pH 9.5 (or if IgG volume is >1mL then increase volume to add to yield same final concentration)
 - c. Leave at 4C for 24 hours

Day 3:

1. Add 0.1mL (or adjusted volume) of 0.2M lysine to IgG-HRP solution.
2. Incubate at room temperature for 2 hours
3. Dialyze overnight against 4 liters of PBS, pH 7.4, at 4C

Day 4:

1. Precipitate HRP-IgG
 - a. Add equal volume of saturated ammonium sulfate, pH 7.0
 - b. Incubate at 4C anytime from 6 hours to overnight
 - c. Spin at 3000g for 30min, at 4C, (or 10,000g in eppendorf tubes)
 - d. Remove supernatant thoroughly with a drawn Pasteur pipette
2. Resuspend pellet in 1mL PBS, pH 7.4
3. Dialyze overnight against 4 liters of PBS, pH 7.4, at 4C

Day 5:

1. Put sample in eppendorf tube
2. Spin ~10 minutes at 10,000 rpm, discard sediment
3. Adjust sample to 1% BSA i.e. add 0.01g ELISA grade BSA to 1mL sample
4. Aliquot and freeze or add 0.02% thimerosal if kept at 4C

Day 5 or later:

1. Test various dilutions of conjugate with the ELISA

Appendix F - Fluorometric DNA assay

This assay is adapted from Labarca and Paigen, Anal. Biochem., 102: 344-352, 1980. It is based on the fact that fluorescence increases upon binding of bisbenzamide to DNA.

Notes:

- ✓ CHAPS lysis buffer does not interfere but other detergents such as Triton X100 and NP40 are incompatible with this assay
- ✓ There is approximately 6pg DNA per cell
- ✓ In order to be in range, from a 35mm dish of McA cells, a suitable volume to assay is 40µL out of 1mL cell extract in CHAPS lysis buffer.

Buffers:

- ✓ CHAPS lysis buffer:
 - (4 mM CHAPS, 50mM NH₄OH, 3U/mL heparin)
 - 615mg CHAPS (4mM final)
 - 850 µL NH₄OH (concentrated) (50mM final)
 - 750 µL heparin (1,000 U/mL) (3U/mL final)
 - 250mL dH₂O
 - pH 8.1 (w/ HCl)
 - just prior to use, add: 1µL cocktail I /mL lysis buffer needed
1µL cocktail II /mL lysis buffer needed
- cocktail I - stock :1mg/mL leupeptin, 1mg/mL antipain, 10mg/mL benzamidine and 10,000 KIU/mL trasylol in trasylol solvent,
- cocktail II - stock: 1mg/mL chymostatin, 1mg/mL pepstatin in DMSO
- ✓ Phosphate buffer:
 - (50mM Na₂HPO₄, 2mM EDTA, 0.02% NaN₃, pH 7.4)
 - 0.1g NaN₃ (alternatively 2mL of 5% NaN₃)
 - 125mL 0.2M Na₂HPO₄
 - 0.372g EDTA (disodium MW 372.24) - alternatively use 2mL of 0.5M EDTA stock
 - Adjust volume to 500 mL
- ✓ NaCl/phosphate buffer:
 - (4M NaCl, 50mM Na₂HPO₄, 2mM EDTA, 0.02% NaN₃, pH 7.4)
 - 116.88g NaCl
 - 0.1 g NaN₃ (alternatively 2mL of 5% NaN₃)
 - 125mL 0.2M Na₂HPO₄
 - 0.372 g EDTA (disodium) – alternatively use 2mL of 0.5M EDTA stock
 - Adjust volume to 500 mL
- ✓ Bisbenzimidazole stock:
 - 200µg/mL in water (bisbenzimidazole hydrochloride, Hoechst 33258, Sigma B-2883)
 - Store at 4C protected from light

- ✓ Calf thymus DNA stock:
0.307mg/mL in 1N NH₄OH (stored in cold room), pH 8.3
Store at 4C

Protocol:

1. Dilution of bisbenzamide:

$$\frac{\text{X}}{100\text{X}} \text{ mL bisbenzamide stock}$$

$$100\text{X} \text{ mL NaCl/phosphate buffer}$$
2. Preparation of standard curve with calf thymus DNA:
 - ✓ Add 32.54μL stock DNA to 1.967mL phosphate buffer.
 - ✓ Use this solution to prepare standards according to the table below. Add as much lysis buffer to the standards as is added for each sample.

Preparation of DNA Assay Standard Curve

<u>μg DNA</u>	<u>Diluted DNA (μL)</u>	<u>Phosphate buffer (μL)</u>	<u>Lysis buffer (μL)</u>	<u>Diluted bisbenzamide (μL)</u>
0	0	750	*	750
0.05	10	740	*	750
0.125	25	725	*	750
0.2	40	710	*	750
0.3	60	690	*	750
0.45	90	660	*	750
0.6	120	630	*	750
0.8	160	590	*	750
1.0	200	550	*	750
1.25	250	500	*	750
1.5	300	450	*	750
1.8	360	390	*	750

3. Prepare samples in at least duplicates (in 10 x 75 mm tubes):
 - 750μL phosphate buffer
 - 750μL diluted bisbenzamide
 - μL* cell lysate (use same volume of lysis buffer for the standards *)
4. Vortex, cover and incubate standards and samples at room temperature for at least 30 min (in the dark). Incubating overnight may increase the stability of the reading.
5. Transfer to black polystyrene, sterile, tissue-culture treated, flat-bottom Costar 96 well plates with lid. For each sample, pipette 300μl per well in triplicates.

- a) Check that the fluorometer is on
- b) Open the Magellan3 program via the desktop shortcut.
- c) Insert the plate into the reader as far towards the upper lefthand corner as possible.
- d) Open the file wizard and click “plate in”
 - “obtain raw data” – “OK” - “next”
 - click “customize measurement parameters”
 - “general” tab: select “fluorescence” radio button
 - “plate” tab: select plate definition GRE96fb.pdf (for these specific Costar plates)
 - “measurement parameters” tab: select excitation = 360nm
emission = 465nm

Use optimal gain at least for reading the first plate. If more plates need to be read under the same conditions as the first, select manual gain, and type in the gain value of the first plate

- Select read mode: top
 - integration time= 40μ sec
 - number of flashes ≤10 – “OK”

- e) Default file will be saved to the specified directory path
- f) Click “start” to read the plate, then “raw data” to see results
- g) To print: click “OK”, then “cancel” until file menu is accessible. Under file menu select “print”
- h) To save as an Excel file: under edit menu select “copy to excel file” (in options, export tab, you can change the orientation of the rows/columns if desired)
- i) To read other plates, go back to the file wizard. Change to manual gain mode and reset gain to the optimal gain value from the readout of the first plate.

Procedure for changing filters in slides:

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Appendix G - Protocol for Cholesterol Analysis by Gas Chromatography

Principle: Free cholesterol (FC) can be assayed directly using the GC, and total cholesterol (TC) can be assayed after saponification of the CE into FC and FA. β -sitosterol (from soybean) is used as an internal standard as it is absent in mammals. (Adapted from Stephen R Behr's PhD thesis, 1983, pp. 40-41)

Internal standard:

Make a 0.5mg/mL stock (10mg in 20mL of chloroform) of β -sitosterol (Sigma #S-9889).

Sample preparation:

1. Rinse the cells with PBS
2. Scrape the cells in 4mM CHAPS lysis buffer (use 600 μ L/35mm dish)
3. Freeze at -20C
4. Sonicate the lysates 2x 20seconds with a ≥ 30 second interval on ice in between
5. Transfer the 600 μ L of sonicated cell lysate in lysis buffer into an acid washed glass screw cap tube (in drawer at the 338 sink)
6. Add 10mL (1 volume) of chloroform : methanol (2:1) and vortex
7. Place on ice for 30 minutes

Note: this step, while not essential, ensures optimal recovery

8. Add 25 μ g β -sitosterol to each tube, that is add 50 μ L of a 0.5 μ g/ μ L in chloroform using a Hamilton syringe (practice dispensing accurate volumes first using solvent only)
9. Add 0.2 volume of water (i.e. 2mL)
10. Vortex well
11. Spin at 1000rpm for 5 minutes (at RT or at 4°C)
12. Discard the upper methanol/water phase. The lower chloroform phase contains the internal standard and lipids
13. At this stage, for each sample, separate this (~6mL) chloroform phase into 2 tubes, one for analysis of TC, one for analysis of FC:
 - a. Transfer 1.5mL to a new acid washed screw-capped flat-bottomed glass tube for analysis of TC (~6 μ g β -sitosterol)
 - b. Keep the rest in original tube for analysis of FC (~19 μ g β -sitosterol)

Analysis of total cholesterol:

- Saponification:
 - Place glass tube in sand bath at ~40-60°C to evaporate the chloroform (cap off)
 - Add 2mL of 2% KOH in 95% ethanol (use pellets, 2g/100mL ethanol)
 - Vortex
 - Place tubes back in sand bath and incubate for another 1hr at 60°C
 - Allow to cool
 - Add 2mL of hexane and vortex
 - Add 2mL of water and vortex
 - Spin for 5 minutes at 1000rpm at RT

- Transfer the upper hexane phase, which contains the saponified lipids, to new glass tubes

Note: because the FA are still in the salt form (basic pH), they are water soluble and will not partition in the hexane phase

- Dry down the hexane phase containing the sterols under nitrogen
- Redissolve in 50µL chloroform
- Vortex well
- Inject 2µL into the GC injection port

Note: expect a signal of around 40-50,000 for cholesterol based on previous trial assays, and a β -sitosterol signal corresponding to 0.24µg

Analysis of free cholesterol:

- Dry the sample under nitrogen gas
- Resuspend in 50µL chloroform
- Inject 2µL into the GC injection port

Note: expect signal of ~16,000 for free cholesterol, and a β -sitosterol signal corresponding to 0.76µg

HP 5890A Gas Chromatograph (GC) settings:

Use a hydrophobic capillary column: RTX-5, 15M Crossbonded SE-54

Restek #10221, Lot# 1649A

Max temperature 325°C

Internal diameter (ID) of 0.32mm

DF=0.25micron

Oven temperature: 220°C

FID and injection port temperatures 270°C, 250°C

Column flow rate: 34.5mL/min

Column head: p=18psi

Split vent: 36.7mL/min and purge vent: 1.1mL/min

Pressure gauges: {air, 41}, {N₂, 43}, {H₂ FID, 19}, {H₂ column, 41}.

Calculations to determine the initial amounts of cell cholesterol:

The Rf (response factor) value for β -sitosterol, the internal standard, was set at 1.

The Rf value for cholesterol was determined to be equal to 1.01.

Knowing that $C_{\text{count}}/C_{\text{mass}} = S_{\text{count}}/S_{\text{mass}} \times R_f$, for each sample the amount of cell free cholesterol (µg/µg DNA) was determined using the following calculation:

$[(FC \text{ cholesterol count} \times 25\mu\text{g}) / (R_f \times \text{sitosterol count})] / \mu\text{gDNA (in 600}\mu\text{L lysate)}$

Similarly, the amount of cell total cholesterol (µg/µg DNA) was determined as follows:

$[(TC \text{ cholesterol count} \times 25\mu\text{g}) / (R_f \times \text{sitosterol count})] / \mu\text{gDNA (in 600}\mu\text{L lysate)}$

Appendix H - Hepatic Lipase Activity Assay

NOTES:

- 1) The final concentration of NaCl in the 0.5mL assay mixture must be 1M, such as the lipoprotein lipase activity is suppressed.
- 2) These assays have a very small range of linearity representing the hydrolysis of only a few percent of the total substrate. Therefore, to be quantitative is best done using the higher (10X) specific activity substrate, assaying replicates at several aliquot levels.
- 3) To be in range, 5-10ng of HL should be assayed.
- 4) For human HL, activity is normally $\sim 10,000 \mu\text{eq/hr/mg}$ (i.e. 10neq/ng). For rat HL, activity is higher, $\sim 40,000 \mu\text{eq/hr/mg}$.
- 5) Control assays (no enzyme) are necessary to determine the effect of all non enzyme components of the sample on the substrate and subsequent partition of the radiolabeled TO and FFA that may arise. One should also consider how these components affect the inhibition or enhancement of the sample lipase activity.
- 6) Preparation of samples to be assayed:
Total assay volume (sample plus substrate mixture) is 0.5mL. Prior to assaying samples, the buffer conditions should be tested for interfering substances (such as CHAPS).

✓ Media Samples

- a -Incubate cells with fresh media for a specified amount of time (including heparin in the medium if desired).
- b -Collect media; centrifuge at 4°C for 30 minutes at 2000rpm (or 10min at 14,000rpm) to pellet cells and cellular debris.
- c -Transfer supernatant; store on ice until needed for assay.

✓ Cell Extract Samples

- a -Grow cells to desired density; place dishes on ice to collect
- b -Rinse cells 1 or 2x with appropriate volume of ice-cold PBS.
- c -Collect cells by scraping in appropriate volume of Lysis Buffer. Of note, 4mM CHAPS lysis buffer does interfere with this assay.
- d -Sonicate cell extracts 2x 30seconds, 100watts using the Braun-sonic 1510 probe sonicator (place cells on ice for 30sec. in between pulses).
- e -Spin supernatant for 10 minutes at 14,000rpm at 4°C .
- e -Store on ice until needed for assay.

✓ Other Lipase Sources

The assays may need to be performed on samples from a variety of sources (i.e. acetone powders, various steps during purification). In general, check for interfering substances, and make sure 1M NaCl/tube is maintained.

REAGENTS:

- 1) **TLC purified ^{14}C or ^3H radiolabeled TO** in chloroform with 50 $\mu\text{g/mL}$ butylated hydroxytoluene. The cpm/g chloroform is determined by liquid scintillation counting.
- 2) **Triolein (TO)**, MW = 885.4, Sigma # T-7140, Sigma Grade. Prepare a solution containing 33.33 μEq TO/g reagent grade chloroform (0.75% ethanol). This is 29.51mg TO or 100 μEq FA/g chloroform.
- 3) **Oleic acid (OA)**, Sigma # O-3879, ~99%.
- 4) **5% Gum Arabic**, from Acacia tree, Sigma # G-9752. Dissolve 15g to a total volume of 300mL with dH₂O. Stir vigorously – may take a long time to completely go into solution. Filter through Whatman #1 paper. Aliquot ~3mL into 5mL screw cap tubes. Store at -20°C.
- 5) **10% BSA, 2M Tris, pH 8.6**. Dissolve 20g of crystalline Bovine Serum Albumin, ICN #810014, and 48.4g of Tris base to ~180mL with dH₂O. Check pH and adjust to 8.6. Adjust volume to 200mL with dH₂O; filter through Whatman #1 (or #114) filter paper. Aliquot ~2.2mL into 5mL screw cap tubes. Store at -20°C.
- 6) **Belfrage "killing solution"**, 4 liters: (all solvents reagent grade)
1541mL methanol or 1227g at density 0.796g/mL (30.8% by weight)
1093mL heptane or 748g at density 0.684g/mL (50.5% by weight)
1366mL chloroform or 2012g at density 1.473g/mL (18.7% by weight)
~400mg oleic acid in chloroform solution (for a 0.1g/L final concentration) is added as a part of the total chloroform, serving as a carrier for the fatty acid released by the HL in the assay. Store at RT
- 7) **Borate buffer**, reagent grade components, 2 liters (aqueous):
152.8g potassium tetraborate (0.25M for MW = 305.5)
69.1g potassium carbonate (0.25M for MW = 138.2)
pH adjusted to 9.5
Store at room temperature
- 8) Glass mini-vial for substrate preparation, 17x58mm, Wheaton # 98649.

PROCEDURE:

- 1) Select:
 - the desired specific activity of substrate (1x which is 50,000ncpm/ μEq FA, or 5x (250,000), or 10x (500,000)),
 - the volume of sample to assay/tube (0-200 μL),
 - and the total number of assay tubes.

Knowing that the total reaction volume is 0.5mL:

$\text{Total volume of substrate (mL)} = \text{number of tubes} \times \frac{0.5\text{mL} - \text{sample volume}}{\text{tube}}$

- 2) Successively weigh out appropriate amounts of (100 μEq FA/g chloroform solution) cold triolein and TLC purified "hot" triolein mixture for the desired specific activity into a mini-scintillation vial. An Excel spreadsheet (with the specific activity value of the current batch of hot triolein, and options for the number of tubes and the desired specific activity) is available and is routinely used to assemble the substrate.

Otherwise, one can calculate the amounts to use such as:

$$\text{Cold triolein (g)} = \frac{\text{number of tubes} \times 3.75 (\mu\text{eq FA/tube})}{100 (\mu\text{eq FA/g})}$$

$$\text{Hot triolein (g)} = \frac{\text{desired specific activity of substrate (ncpm}/\mu\text{eq FA)} \times \text{number of tubes} \times 3.75 (\mu\text{eq FA/tube})}{\text{specific activity of batch of hot triolein (ncpm/g)}}$$

Note: no matter what the desired specific activity is, the mass of cold triolein is the same (such as there is 3.75 μeq/tube), and the mass of the labeled TO (which increases with increasing specific activity) is always assumed to be a negligible fraction of the total mass of cold TO.

3) Evaporate chloroform with a gentle stream of N₂ (takes a few minutes). The last stage of this evaporation requires manipulating the stream of N₂ in a fashion that "moves" the visible TO mass around the bottom perimeter of the vial. This is necessary in order to remove the last traces of chloroform entrapped in the TO mass.

4) Add appropriate volume of the 5% gum arabic solution to the substrate mix to reach a 0.5% final concentration in the total assay reaction volume (i.e. add 0.05mL/tube).

From this point on, make sure substrate mixture is kept on ice.

5) Sonicate 3x 30 second cycles at 100 watts using the Braun-sonic 1510 probe sonicator with 4mm OD microtip. In between cycles, return the mixture to an ice bath for at least 30 seconds. This sonication step is most important in the substrate preparation. One must be certain to sonicate rather than cavitate (stir air into the mix). Cavitation is most likely to occur if the tip is removed from the solution while power is applied to the tip. Avoid touching the glass with the probe. Large droplets of TO remaining on the surface of the emulsion indicate that the preparation was not sufficiently sonicated.

6) Add appropriate volumes of 10% BSA-2 M Tris pH 8.6 to the substrate mix to reach 1% BSA and 0.2M Tris in the final assay reaction (i.e. add 0.05mL/tube). At this point, transfer the substrate mix from the glass vial to a 50mL Falcon tube

7) Add appropriate volume of 4M NaCl to the substrate mix – use the solution to rinse the glass vial and transfer to the Falcon tube. If sample volume is negligible and/or if sample is 1M NaCl, then volume of 4M NaCl to add is total volume of substrate mix /4 in order to reach 1M NaCl final – otherwise, sample salt concentration needs to be taken into account:

$$\text{Molarity substrate} = [0.5\text{mL} \times 1\text{M NaCl} - (V_{\text{sample}} \times \text{Molarity sample})] / V_{\text{substrate}}$$

$$\text{Vol. of 4M NaCl to add} = (V_{\text{substrate}} (\text{mL}) \times \text{Molarity substrate}) / 4 (\text{M NaCl})$$

8) Add appropriate volume of water

Vol. H₂O (mL) = number of tubes x ((0.5mL - sample volume)/tube)
- Vol of gum Arabic - Vol of (BSA/Tris) - Vol of NaCl

9) Cap and mix gently by hand, then aliquot substrate mix to each 13 x 100 mm glass disposable assay tube, keep on ice

10) Optional preincubation of samples: add control or anti-HL serum to each sample. (Be sure to add the serum directly into the sample and not on the wall of the tube). Cover tubes with saran wrap. Shake at 4°C on ice for 45 minutes immediately prior to assay.

11) Enzyme Assay:

- a - Place substrate aliquots on ice (or enzyme/serum solution if preincubation step is needed) in 13x100mm glass tubes.
- b - Add enzyme solution (or substrate) to the first tube; vortex briefly and begin timing as the tube is placed in a 30°C H₂O bath with constant agitation.
- c - At timed intervals, repeat step b for the remaining samples. Optimum assay time will depend on the sample and substrate specific activity – common assay times are 1/2hour or 1hour.
- d - Stop reaction (at timed intervals) by adding 3.25mL (6.5 volumes) Belfage Killing solution and 0.75mL (1.5 volume) Borate Buffer, pH 9.5. Vortex vigorously for 10-20 seconds. Do NOT replace tubes on ice as this will affect the partition coefficient.
- e - Spin for 10 minutes, room temperature, 800xg (i.e. 2000rpm @ a radius of 18cm in a Model UV IEC centrifuge).
- f - Carefully transfer 1.9mL (P1000 pipetman) of upper layer into 9mL Liquiscint liquid scintillation solution in a 20mL glass scintillation vial, mix and count.

12) Liquid scintillation counting and calculations:

Values are usually determined in units that reflect µEq FA hydrolysed by the lipase per mL of sample per hour. In order to correctly calculate this value it is essential to define:

- the cpm in the assay tube aliquot
- the partition fraction of fatty acid in the upper layer, as established in a "blank" assay by replacing the radiolabeled TO with a known trace of radiolabeled oleic fatty acid.
- the absolute counting efficiency of the liquid scintillation counter should be defined for each assay using a known dpm standard prepared in 1.9mL of a blank assay upper layer and 9mL counting cocktail. This allows actual dpm to be used in the calculation or for a correction to cpm due to changes in counting efficiency
- the volumetric fraction of the upper layer taken to count
- the specific activity of the substrate, defined as the cpm (or dpm) TO/µEq TO in the substrate, according to the proportion of hot and cold TO used in making the substrate.

13) Final calculation of catalytic activity:

$\frac{\mu\text{Eq FA / hour / mL sample}}{\text{netcpm} \times 2}$
$\frac{\text{volume(mL)} \times \text{incubation time(hr)} \times 0.38 \times \text{substrate specific activity(cpm}/\mu\text{Eq FA)} \times 1.9}{\text{netcpm} \times 2}$

where :

- volume is the volume of lipase sample assayed
- 2/1.9 accounts for the fact that only 1.9mL are counted instead of 2mL,
- 0.38 is the recovery fraction of FA in 2mL of upper layer,
- cpm could also be replaced by dpm.

As a first estimate, one can divide the net cpm (sample gross cpm – buffer blank gross cpm) in $\mu\text{eq/hr}$ by either 10,000 (for human HL) or 40,000 $\mu\text{eq/hr/mg}$ (for rat HL) to obtain an approximation of the mass of normally active lipase present in the sample (in mg).

An ELISA assay performed on the same samples can provide a reliable mass value for HL in the sample, in $\mu\text{g/mL}$. By dividing the activity value ($\mu\text{mol/hr/mL}$) by the mass ($\mu\text{g/mL}$), one can now obtain the specific activity of the lipase in $\mu\text{mol FA/hr}/\mu\text{g}$.

Appendix I - RNA Isolation Using Trizol™ and RNeasy columns

1. Trizol (Invitrogen) extraction

The yield is generally ~100-300µg RNA/100mm dish for confluent McA-RH7777 cells, or ~30-45µg RNA/35mm dish. The procedure below is for isolation from 35 mm dishes, double the volumes for isolation from 100mm dishes.

a. HARVEST

- ❖ Add 1.25mL Trizol™ to each 35mmdish to lyse the cells
- ❖ Pipette up and down a few times to homogenize
- ❖ Transfer the lysates to RNase free eppendorf tubes
- ❖ Incubate for 5 minutes at RT

It is optional to store at –80°C at this step.

b. PHASE SEPARATION

- ❖ Add 0.25mL chloroform, which is 0.2mL/mL Trizol™
- ❖ Shake the tube vigorously by hand for 15 seconds
- ❖ Incubate for 3 minutes at RT
- ❖ Spin at 11,300 rpm (12000g) for 15 minutes at 4°C

c. RNA PRECIPITATION

- ❖ Transfer the upper aqueous phase to clean eppendorfs
- ❖ Add 0.5mL isopropyl alcohol per 1mL Trizol™, which is 0.625mL/tube
- ❖ Mix by pipeting up and down
- ❖ Incubate the samples for 10 minutes at RT
- ❖ Centrifuge at 11,300 rpm for 10 minutes at 4°C (The gel like pellet that will form is the RNA)

d. WASH

- ❖ Remove the supernatant and wash the pellet with at least 1.25mL/tube of 75% ethanol. Do NOT vortex
- ❖ Spin at 9000 rpm (7500g) for 5 minutes at 4°C
- ❖ Remove the supernatant
- ❖ Air dry
- ❖ Resuspend the RNA in 100µL RNase-free water

2. Optional: RNeasy columns (QIAGEN)

The RNeasy kit further purifies the RNA and very good (almost 100%) recoveries are achieved.

RNA isolated from a single 35mm dish or a pool of 2 dishes can be loaded directly onto an RNeasy column (QIAGEN). If RNA is extracted from more cells, the RNA mass needs to be measured first so that no more than 100µg (the maximum capacity of the column) are loaded on the column.

Follow the manufacturer's instructions.

Appendix J - Real-time RT-PCR using the TaqMan probe assay

Basic principle: The TaqMan assays use a fluorogenic probe that anneals within the cDNA target sequence during PCR. Because the probe is designed to have a lower annealing temperature, it anneals prior to annealing of the primers. A reporter dye (FAM) is attached to the 5' end of the probe, and a quencher dye is attached to its 3' end. During PCR, the 5' nuclease activity of the DNA polymerase cleaves the probe, which releases the reporter dye from the quencher and increases the fluorescence intensity.

Preparation of RNA samples:

RNA was isolated using the Trizol reagent (Invitrogen) and (optionally) further purified using the RNeasy mini kit (Qiagen).

Reagents for real-time RT-PCR (Applied biosystems):

<u>Product # :</u>	<u>Description:</u>
4311235	Multiscribe RT, 100µL
N808-0260	dNTP mixture, 1mL
N808-0128	Oligo(dT), 100µL
4324018 1	TaqMan Universal PCR mastermix, 5mL
N808-0234	TaqMan RT reagents for 200 reactions

Reverse transcription:

1 or 2µg RNA was reverse transcribed using oligo dT as primers and MultiScribe™ Reverse Transcriptase (Applied Biosystems).

(Note: this is a recombinant Moloney murine leukemia virus (rMoMuLV) reverse transcriptase)

Make one blank reaction for no template control (NTC).

Pick either a 50µL (1µg RNA) or 100µL (2µg RNA) reaction format and calculate volumes to be added to the master mix accordingly.

Component	µL per reaction	µL per reaction	µL for master mix	final concentration
10X TaqMan RT Buffer	10	5		1X
25 mM MgCl ₂	22	11		5.5 mM
dNTPs (2.5mM each)	20	10		500 µM/dNTP
oligo dT	5	2.5		2.5 µM
RNase inhibitor	2	1		0.4 U/µL
Multiscribe RT (50 U/µL)	2.5	1.25		1.25 U/µL
water + RNA	38.5	19.25		

100 50

Aliquot 61.5µL/tube for a 100µL reaction or 30.75µL/tube for a 50µL reaction in 0.3mL PCR tubes for the robocycler.

After making aliquots of the master mix, to each tube, add:

- 2µg RNA and water to a total volume of 38.5µL if running a 100µL reaction
- 1µg RNA and water to a total volume of 19.25µL if running a 50µL reaction

Mix well

Anneal primers by incubating for 10 minutes at 25°C (room temperature)

In robocycler, do the reverse transcriptase reaction at 48°C for 30 minutes, then do a 5min incubation at 95°C to inactivate the enzyme

Freeze cDNA at -20°C until real-time PCR is performed

Real time polymerase chain reaction:

Real-time PCR using cDNA as a template was performed utilizing a custom-designed Taqman probe assay (Assays-by-Design(SM) Service, Gene Expression, Assay ID 4331348) with two unlabeled PCR primers (SENDAK_RHL-E2E3F :CCA TCC ACT TGT CAT GAT CAT CCA and SENDAK_RHL-E2E3R : CTT CCA GAT CCA GGT TTC TAG CAA, 18µM each) and a FAM™ dye-labeled TaqMan® MGB probe annealing on the same strand as the reverse primer (FAM CAT CCA CCG ACC ACC C, 5µM). The assay and cycling conditions were set according to the manufacturer's instructions.

Notes about the cDNA sequence and the design of the probe:

The probe was designed based on the corrected cDNA sequence from Sendak. The exon/intron junctions are not known for rat, but only those exon/exon junctions that bore 100% homology with the human sequence were sent as options for the design of a probe. The designed probe overlaps the putative junction of exon2 and exon3.

Calculations for standard:

Calculations of rHL plasmid DNA standard using the following molecular weights:

A	C	G	T
267.2	243.2	283.2	242.2

 g / mole

The rHL cDNA is inserted in pCDNA3.1 (-)

	Number of each base				MW/ strand	Total MW	
	A	C	G	T			
rHL insert: sense	446	413	389	354	415516	829693	Sendak cDNA sequence
anti-sense	354	389	413	446	414176		
pcDNA3.1(-): sense	1253	1414	1382	1378	1403820	2812046	Invitrogen website
anti-sense	1378	1382	1414	1253	1408225		
						3641739	= MW of vector & insert

1 mole =6.022E+23 copies

The number of rHL transcripts was calculated using the following formula:

$$\text{rHL transcript number} = (\text{rHL standard mass in pg}) \times 10^{-12} \times (6.022\text{E}+23) / \text{MW}$$

where MW=3641739

Reaction:

- For HL, aliquot 16µL master mix to all wells being used, then add 4µL/well of either standard, blank cDNA for the no template control (NTC) wells, or cDNA sample.
- For internal standards such as P0, first dilute the cDNA 1:9 in water, then use 9µL of this diluted cDNA in the 20µL reaction.

Use filter tips for all pipetting and sterile mQ water in baked glassware for the master mix and standard dilutions.

Component	Vol. (µL) per HL reaction	Vol. (µL) per internal standard reaction
Taqman 2X PCR Master Mix	10	10
20X assay mix	1	1
water	5	0
standard or cDNA	4	9 from 1:9 dilution
Total volume (µL)	20	

Cover the plate with an optical adhesive cover when finished. Do not write on the plate. Spin down plate (remaining air bubbles aren't problematic as they will disappear once the machine temperature increases).

Run plate on the ABI Prism® 7900HT thermocycler. Use SDS 2.0 software with "Absolute quantification" for "384 well plate" and rHL FAM as detector. The ABI kit uses ROX fluorescence as a passive reference for background. Real time thermal profile should have 2 steps, first step should be 1x (95C, 10 minutes), stage 2 should be 40x {(95C, 15 seconds), (60, 1 minute)}.

Appendix K - Luciferase assays

Principle/chemistry of the assay:

A promoter fragment of the gene under investigation is cloned in an experimental vector upstream of a reporter gene to drive its expression. The “experimental” reporter is correlated with the effect of specific experimental conditions, while the activity of the co-transfected “control” reporter provides an internal control to account for sources of variability such as differences in cell viability or transfection efficiency, differences in pipetting volumes, cell lysis efficiency and assay efficiency.

In the Dual-Luciferase® Reporter (DLR™) Assay, the activities of firefly (*Photinus pyralis*) and Renilla (*Renilla reniformis*, also known as sea pansy) luciferases are measured sequentially from a single sample. Firefly and Renilla luciferases, because of their distinct evolutionary origins, have dissimilar enzyme structures and substrate requirements. The firefly luciferase reporter is measured first by adding Luciferase Assay Reagent II (LAR II) to generate a stabilized luminescent signal. Immediately after quantifying the firefly luminescence, the Stop & Glo® Reagent is added to the same tube. Within 1 second of addition, this reagent simultaneously quenches the luminescent signal from the firefly reaction by at least a factor of 105 and completely activates the Renilla luciferase reaction. The amount of light integrated over a certain period of time is proportional to the amount of luciferase reporter activity in the sample, which in turn reflects the activity of the promoter driving its expression.

Light intensity is a measure of the rate of catalysis by the luciferases. In the Firefly luciferase reaction, photon emission is achieved through oxidation of beetle luciferin in a reaction that requires ATP, Mg^{2+} and O_2 . The luminescent reaction catalyzed by Renilla luciferase utilizes O_2 and coelenterateluciferin (coelenterazine). The kinetics of the Renilla luciferase reaction provide a stabilized luminescent signal that decays slowly over the course of the measurement.

The pRL-TK vector expresses Renilla luciferase under the control of the herpes simplex virus thymidine kinase promoter. As an internal control, it should be unresponsive to treatment. However, this vector has been shown to vary upon treatment with adenoviral 12S E1A oncoprotein in Saos-2 cells (Thavathiru and Das, *BioTechniques* **31**: 528-32) or dihydrotestosterone and dexamethasone in CV-1 cells (Ibrahim et al., *BioTechniques* **29**: 782-4). Thus, for the study of some transcriptional regulators, the pRL-TK vector is not an appropriate control. If the internal control expression is affected by the treatment, it may be more accurate to rely on data from statistical analyses of replicates in multiple experiments.

Standard Protocol

Materials: Promega Dual-Luciferase Reporter Assay System

1. Typically, rat hepatoma cells were transfected in 24 well plates with 80ng/well total DNA, with a 10:1 molar ratio of experimental luciferase reporter to pRL-TK control vectors respectively. Transfection was done in a total volume of 250 μ L DMEM (no serum) in the presence of 1 μ L lipofectamine and 2 μ L Plus reagent (Gibco life technologies) according to the manufacturer's instructions. Three hours after transfection, the medium was replaced and the appropriate maintenance or treatment medium added to the cells
2. Freshly prepare a sufficient quantity of the 1X passive lysis buffer (PLB) by diluting the 5X concentrate (stored at -20°C) in distilled water. Mix well.
3. Remove the growth medium from the cultured cells, and gently rinse the cells with phosphate buffered saline (PBS). Aspirate the PBS completely.
4. Dispense 1X PLB into each culture well (100 μ L/well for a 24-well culture plate, 65 μ L/well for a 48-well culture plate, and 20 μ L/well for a 96-well culture plate)
5. Shake the culture plates gently at RT on a rocking platform for 15 minutes to ensure complete and even coverage of the cell monolayer with 1X PLB. Optionally freeze the plates at -80°C to achieve active lysis. The firefly and Renilla luciferases in PLB are stable at -70°C for over one month. The lysis buffer can be pipetted directly from the wells of the culture plate for the assay.

The assays for firefly luciferase activity and Renilla luciferase activity are performed sequentially in a single reaction tube.

6. Reagents preparation:
Luciferase Assay Reagent II (LAR II): resuspend the provided lyophilized Luciferase Assay Substrate in 10mL of the supplied Luciferase Assay Buffer II. LAR II is stable for one month at -20°C or for one year when stored at -70°C . Repeated freeze-thawing of this reagent may decrease assay performance. The components of LAR II are heat-labile, thaw in a water bath at room temperature. Mix the thawed reagent prior to use.
Stop & Glo® Reagent: Make enough for 100 μ L per assay. Reconstitute the 50X Stop & Glo® Substrate in 50 volumes of Stop & Glo® Buffer in a glass or siliconized polypropylene tube. Stop & Glo® Reagent (Substrate + Buffer) is best when prepared just before use, but can be stored at -20°C for several weeks.
7. Predispense 100 μ L of LAR II into the appropriate number of luminometer tubes (12x75mm borosilicate glass tubes)
8. Program the LUMAT single-cell luminometer to integrate the luminescence over 10 seconds.
9. Perform a first reading with just the tube and LARII
10. Add 20 μ L of cell lysate to the tube; mix by pipetting 2 or 3 times, and read again.
11. Add 100 μ L of Stop & Glo® Reagent and vortex briefly to mix. Read.
12. Discard the reaction tube, and proceed to the next DLR™ Assay.

Protocol for use in 96 well plates using the Dual-Glo™ Luciferase Assay System

Materials: Dual-Glo™ Luciferase Assay System (#E2920):

Note: When pipeting in a 96 well plate, always pipet on the side of the wells such as not to disturb the cell monolayer. Use a 12-channel pipetor.

1. Collagen coat (50µL/well at 25µg/µL) white-sided, clear bottom, tissue-culture treated, polystyrene 96 well plates (Costar 3610), and expose to UV light ON (this step is actually critical to have a nice cell monolayer with McA cells)
2. One day before transfection, plate cells in 100µL medium at 40,000cells/well.
3. Transfection of cells: Typically, McA/Fu5AH cells in 96 well plates were transfected with 25ng/well total DNA, with a 50:1 molar ratio of experimental luciferase reporter to phRL-TK control vector respectively. Transfection was done in a total volume of 70µL MEM in the presence of 0.5µL Lipofectamine and 1µL Plus reagent (Gibco life technologies) according to the manufacturer's instructions. Three hours after transfection, the medium was replaced and 100µL of the appropriate maintenance or treatment medium was added to the cells.
4. Luciferase assays were performed 36 – 48 hours after transfection.
 - a) **Reagent preparation:**
 - Reagents (stored at –20C) should be equilibrated to room temperature (in water bath) before beginning measurements. Do not thaw at T> 25°C. Mix well.
 - Transfer the contents of one bottle of Dual-Glo™ Luciferase Buffer to one bottle of Dual-Glo™ Luciferase Substrate to create the Dual-Glo™ Luciferase Reagent. Mix by inversion until dissolved. Can be stored at –70°C for up to one month.
 - Calculate the amount of Dual-Glo™ Stop & Glo ® Reagent needed to perform the desired experiments. Immediately prior to use, dilute the Dual-Glo™ Stop & Glo ® Substrate 1:100 into Dual-Glo™ Stop & Glo ® Buffer.
 - b) **Cells preparation:** Remove plates containing mammalian cells from the incubator. Aspirate medium and add 50µL MEM back to each well. Equilibrate cells in media to room temperature before performing luciferase measurements.
 - c) **XFluor4 setup:** Select “edit measurement parameters”, “luminescence”, “no kinetics”, integration time of 5000ms, gain of 200 as a start (from 1-255, can be optimized), “no shaking”, “read from top”, “Costar 96ft.pdf” for Corning Costar 3610 plates. Remove the emission slide for the duration of the assay. Insert the tissue-culture plate in the reader and place the black insert over it to minimize cross talk in between wells
 - d) **Measure firefly luciferase activity:** Add a volume of Dual-Glo™ Luciferase Reagent equal to the culture medium volume to each well (50µL) and mix. This reagent induces cell lysis and acts as a substrate for firefly luciferase, producing a stable luminescent signal for two hours. Wait at least 10 minutes (but not more than 2 hours), then measure the firefly luminescence by selecting “start measurement” under the XFluor4 menu.
 - e) **Measure Renilla luciferase activity:** Add a volume of Dual-Glo™ Stop & Glo ® Reagent equal to the original culture medium volume to each well (50µL) and mix. This reagent quenches the luminescence from the firefly reaction by at least 10,000-fold and provides the substrate for Renilla

luciferase. Wait at least 10 minutes (but not more than 2 hours), then measure renilla luminescence (in the same plate order as the firefly luminescence when appropriate).

Calculations:

$$\text{Firefly}_{\text{corr.}} = \text{Firefly count} - (\text{average of } \sim 3 \text{ firefly readings from untransfected wells}) \\ \approx \text{Firefly count} - (\text{background count (LARII reading) for same tube})$$

$$\text{Renilla}_{\text{corr.}} = \text{Renilla count} - (\text{average of } \sim 3 \text{ Renilla readings from untransfected wells})$$

$$\text{Firefly to Renilla ratio} = \text{Firefly}_{\text{corr. average}} / \text{Renilla}_{\text{corr. Average}}$$

Any effect of the treatment on the pGL3 basic vector (without an inserted promoter) can be corrected for by expressing the results as a relative increase/decrease of the promoter construct expression upon treatment relative to the relative increase/decrease expression of the pGL3basic upon similar treatment.

$$\text{Normalized ratio} = \frac{\text{Firefly experim corr. average} / \text{Renilla experim corr. average}}{\text{Firefly basic corr. average} / \text{Renilla basic corr. average}}$$

Appendix L - Lowry-Bensadoun Protein Assay

This protocol is an adaptation by Bensadoun and Weinstein (Assay of proteins in the presence of interfering materials, *Anal. Biochem.*, **70**: 241-50, 1976) of the original Lowry et al. procedure (*JBC*, **193**: 265-75, 1951) procedure to assay protein in the presence of interfering materials such as lipids.

Buffers/reagents:

- Lowry A (500mL): 10g Na₂CO₃, 2g NaOH, 0.1g NaK tartrate
- Lowry B (500mL): 2.5g CuSO₄·H₂O
- Folin-Ciocalteu's phenol reagent
- 2% deoxycholate
- 100% trichloro-acetic acid (100g TCA, add water to 100mL final)
- Standard: 100µg BSA/mL H₂O (stock solution frozen in aliquots)

DOC/TCA precipitation

Bring the samples and standards to 0.675mL with H₂O- for standards see table below:

BSA mass	Volume BSA stock	Volume d H₂O
0 µg - blank	0 µL	675 µL
2 µg	20 µL	655 µL
5 µg	50 µL	625 µL
10 µg	100 µL	575 µL
20 µg	200 µL	475 µL
30 µg	300 µL	375 µL

Add 5µL of 2% deoxycholate

Add 0.075mL of 100% TCA. Vortex. Optional: incubate on ice for 20-30 minutes.

Spin at 12,000 rpm for 10 minutes, 4C

Aspirate supernatant with drawn Pasteur pipette (white pellets will be variable in size)

Lipid extraction (optional, only for lipid bound protein)

Add 600µL of ethyl ether:ethanol (3:1) (mix 15mL ether with 5mL ethanol in scintillation vial and seal with parafilm, store at 4C). Vortex.

Spin at 12,000 rpm for 10 minutes, 4C

Aspirate supernatant

Dry completely in vacuum dessicator

Lowry

Freshly mix Lowry A with Lowry B (50:1) to make Lowry C (e.g. 12.5mL with 0.25mL).

Add 1mL Lowry C to each sample/standard. Vortex. Incubate at room temperature for 10 minutes. Make sure pellet has dissolved.

Dilute Folin-Ciocalteu reagent 1:1 (v/v) with dH₂O

Add 100µL F-C solution per sample/standard. Vortex and incubate in the dark at room temperature for 40 minutes to let color develop.

Add 300µL/well in Costar 96 well plate and read at 740nm using the ELISA reader.

Plot using linear standard curve.

Appendix M - Mobility Shift Assay

This protocol is adapted from Current protocols in molecular biology, pp.12.2.1-11, and from Li et al., BBA, 2004, 1679: 141-55.

This protocol can be divided into four stages:

1. preparation of a ^{32}P -labeled DNA probe containing a specific protein binding site;
2. preparation of a nondenaturing gel;
3. binding reaction in which a protein mixture is bound to the DNA probe;
4. electrophoresis of protein-DNA complexes through the gel, and autoradiography.

1. DNA probe preparation

DNA fragments from 10-20 to 300bp long may be used as probes. Longer fragments are likely to contain multiple protein-binding sites, making interpretations more difficult.

- a. Get reverse phase cartridge purified complementary oligos to use as probes (5'OH ends)
- b. Order $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$, (3000Ci/mmol, 500 μCi , 10mCi/mL, Perkin Elmer #BLU502A500UC)
Order 250 μCi /4 probe labeling reactions.
- c. Resuspend oligos in mQ H_2O at:
 - 100 μM stock: resuspend X nmol primer in 10X μL mQ H_2O
 - 10pmol/ μL (i.e. 10 μM) stock, i.e. 1:10 dilution from 100 μM stock
- d. Prepare 5pmol/ μL and 1pmol/ μL double-stranded cold probes to use as specific competitors:
 - Mix reverse (10pmol/ μL stock) and forward (10pmol/ μL stock) primers of each set at a 1:1 molar ratio
 - Anneal equimolar amounts of complementary oligos: (95C, 2min), (37C, 30min)
 - Dilute double stranded probe 1:5 to reach 1pmol/ μL final
- e. Preheat 2 heat blocks at 37C and 95C respectively
- f. End-label single-stranded complementary probes with $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ and T4 polynucleotide kinase (NEB M0201S, 500U at 10U/ μL):
 - In a 0.5mL eppendorf, mix:

Synthetic oligonucleotide (10pmol/ μL)	0.5 μL
10X bacteriophage T4 polynucleotide kinase buffer	1 μL
$[\gamma\text{-}^{32}\text{P}]\text{-ATP}$, 20pmol	3 μL
mQ H_2O	4.5 μL

At this point, mix well by tapping the tube, then add:

Bacteriophage T4 polynucleotide kinase (10U)	1 μL
--	-----------------

Mix well

 - Incubate 1hr at 37C
 - Stop the reaction by adding 1 μL of 0.5M EDTA
 - g. Anneal equimolar amounts of labeled complementary oligos: (95C, 2min), (37C, 30min)
 - h. Purify probe on a nondenaturing polyacrylamide gel.

Prepare the gel

- The migration distance (D) of double-stranded DNA through a nondenaturing gel is $D \approx -\log(MW)$ (depends slightly on the sequence). Prepare the gel solution (1X TBE, 10% such as Bromophenol blue will migrate to ~30bp). For a nondenaturing 10% polyacrylamide gel of 18 cm × 16 cm × 1.5 mm, make 60mL (6mL 10× TBE buffer, 20mL of 30% Protogel (37.5:1 acrylamide/bisacrylamide), 33.5mL H₂O). Mix. Add 400μl of 10% (w/v) APS (stock <1 month old stored at 4°C) and 30 μl TEMED and mix thoroughly. Pour and insert the comb.
- Allow the gel to polymerize for ~30 min
- After polymerization is complete, remove the comb and wash the outside of plates. Fill the lower and upper reservoirs of the electrophoresis tank with 1× TBE buffer, and remove air bubbles trapped under the gel, if any.
- Prerun and warm the gel for at least 30 min at 5V/cm (constant voltage) (~100V).

Run the gel

- Add 10× loading buffer to DNA samples (to 1× final). Load.
- Run the gel at ~2-10 V/cm, taking care to avoid excessive heating. If needed to avoid overheating, shorter electrophoresis times may be achieved by using a fan or running the gel at higher voltages in a cold room
- Turn off the power supply. Carefully pry apart the plates so that the gel is still attached to one plate. Wrap the gel and plate with plastic wrap. Expose to X-ray film (~10 seconds) to yield a thin grey band. Make sure orientation is known.
- After exposure, develop the film
- Use the film to precisely localize the area of the gel that contains the full-length probe (most slowly migrating most intense band).
- Align film and gel. Invert and circle the band on the glass plate.
- Turn the gel back over and excise the band using a razor blade
- Transfer the band with clean forceps to a microfuge tube.

Elute the DNA from the gel

- Crush gel with p1000 tip (gel is quite brittle)
 - Add ~2 volumes elution buffer (10mM Tris, 50mM NaCl, 1mM EDTA)
 - Incubate at 37C for 4 hours for fragments <500bp, vortexing regularly if possible
 - Wrap with parafilm and spin at 12,000g for 1minute
 - Transfer supernatant to a new screw cap tube and rinse pellets once or twice (<0.5mL total)
 - Add 5μL Glycoblue, 1/10th volume of 3M sodium acetate, and 2 volumes of ice-cold ethanol
 - Place overnight at -20C
 - Spin at 12,000g for 20minutes, 4C to pellet
 - Rinse with 70% EtOH – spin again and discard supernatant (repeat if necessary)
 - Air-dry
 - Resuspend in 250μL TE, pH 8.0
- i. Count 1μl for Cerenkov counts in a scintillation counter to determine specific activity (cpm/μl). Store probes at 4C for up to 2 weeks. Avoid repetitive freeze/thaw cycles.

2. Prepare the nondenaturing gel

- Dilute 10× electrophoresis buffer 1:20 to prepare 0.5X TBE electrophoresis buffer
- Assemble washed glass plates and 1.5mm spacers for casting the gel. All detergent must be removed to avoid disrupting protein-DNA interactions.
- Prepare 60mL of nondenaturing gel mix/gel (4% in 0.5X TBE – made fresh):
 - 3mL 10X TBE electrophoresis buffer
 - 8mL of Protogel (30% - 37.5:1w/w acrylamide/bisacrylamide)
 - 1.875mL of 80% glycerol
 - 46.57mL mQH₂O
 - Add 400μL of 10% ammonium persulfate and 30μL TEMED
- Immediately pour the gel mix between the plates and insert a comb. Allow the gel to completely polymerize for 20 min. Use a comb with teeth that are ≥7 mm wide.
- Remove any air bubbles trapped beneath the gel and flush out the wells.
- Prerun the gel 30 to 60 min at 100 V.

3. Prepare the binding reactions

Thaw frozen extract aliquots on ice.

Option 1: Regular/competition Mobility Shift Assay

- Add PMSF to 10X binding buffer (500mM KCl, 100mM HEPES, pH 7.9, 1mM EDTA) at 2.5mM final in 10X buffer
- While the gel is prerunning, assemble the binding reaction by combining the following in a 0.5 or 1.5mL microcentrifuge tube:

Amounts in μL	Free probe	Probe + extract	Probe + extract + cold specific probe	Probe + extract + cold non-specific probe
Water	9.5	To 20	To 20	To 20
80% glycerol	2.5	2.5	2.5	2.5
Cold probe (1pmole/μL)	0	0	1	1
10X binding buffer	2	2	2	2
1μg/μL poly(dI:dC)-poly(dI:dC)	2	2	2	2
3μg/μL 10X BSA stock	2	2	2	2
10μg crude extract (last)	0	<3	<3	<3
10,000cpm/μL ³² P probe	2	2	2	2
Total	20	20	20	20

- Mix gently by tapping the bottom of the tube with a finger without introducing air bubbles.
- Incubate at 10-15C for 20minutes in a constant-temperature water bath.
- Proceed to running the gel

General notes:

- A typical binding reaction will contain about 5,000-20,000cpm and about 10-100fmol probe (10fmol/10μL i.e 1nM).
- Amount of DNA and protein must be titrated (0.1 to 20μg crude extract for titration), usually need up to a 5 fold molar excess of binding protein to DNA
- One usually needs 0.1-2μg poly dI /2-3μg crude protein

- For crude extracts, use incubation at 4-15°C, up to 25°C. Optimal incubation temperatures (up to 37°C) for different proteins can vary. Incubate up to one hour.
- To assess the sequence specificity of protein-DNA interactions, the nonspecific competitor can be essentially any fragment with an unrelated sequence, but it is useful to roughly match the probe and specific competitor for size and configuration of the ends. Perhaps the best control competitor is the probe with a mutation in the binding site. Assuming there is not a huge excess of binding protein to probe, typical amounts of competitor are 10-1000X molar excess relative to the labeled probe.

Option 2: Antibody Supershift Assay

If the protein that forms the complex is recognized by the antibody, the antibody can either block complex formation, or it can form an antibody-protein-DNA ternary complex and thereby specifically result in a further reduction in the mobility of the protein-DNA complex (supershift).

- Add PMSF to the 10X antibody binding buffer (1M NaCl, 100mM HEPES, pH 7.9, 1mM EDTA, 30mM MgCl₂, 1mg/mL BSA) at 2.5mM final in 10X buffer
- Assemble the binding reaction:

Amounts in μL	Free probe	Probe + extract	Probe + extract + rabbit IgG	Probe + extract + rabbit antibody	Probe + extract + goat IgG	Probe + extract + goat antibody
Water	11.7	To 18	To 18	To 18	To 18	To 18
80% glycerol	2.5	2.5	2.5	2.5	2.5	2.5
10X antibody binding buffer	1.8	1.8	1.8	1.8	1.8	1.8
1 μ g/ μ L poly (dI:dC)-poly (dI:dC)	2	2	2	2	2	2
10 μ g crude extract	0	<3	<3	<3	<3	<3
2 μ g appropriate antibody	0	0	1-10	1-10	1-10	1-10
Total	18	18	18	18	18	18

- Tap to mix and incubate at 4°C for 2 hours
- Add 2 μ L/tube of 10,000cpm/ μ L ³²P probe
- Incubate at 10-15°C for 20 minutes while pre-running the gel
- Proceed to running the gel

General notes:

- Start with a 1:1 molar ratio antibody to protein – titrate antibody if necessary.
- Results may be different depending upon whether the antibody is added before or after the protein binds DNA (particularly if there are epitopes on the DNA-binding surface of the protein).

4. Run the gel

- a. Load each binding reaction into a well of the pre-ran gel. Load a small volume of 10X loading buffer with dyes into a separate well, or add it only to the negative control sample without extract. There is no stacking gel in this system; so precise loading with little mixing with the gel buffer is necessary to obtain sharp bands. Allow the sample to fall along one side of the well and avoid bubbles.
- b. Electrophorese at 35mA for the minimum time required to give good resolution. Stop the gel before the bromophenol blue approaches the bottom of the gel (~1hr for a 16-cm gel). Longer run times may cause a weaker signal due to partial dissociation of complexes during electrophoresis. Place gel apparatus in cold room or use a fan if glass plates become more than slightly warm.

5. Analyze the gel

- a. Slowly open and remove a glass plate with a spacer, using water to help if needed
- b. Wrap the gel on one plate with plastic wrap and directly expose to a phosphorimager screen at room temperature (~1-24hours in general). If immediate exposure is not possible, force gel to lie flat (e.g. in a cassette).

Buffers and materials/chemicals:

10X TBE (Tris/borate/EDTA) electrophoresis buffer:

0.89M Tris	108g Tris base
0.89M boric acid	55g boric acid
20mM EDTA	40mL 0.5M EDTA pH 8.0
mQ H ₂ O to 1L	

10× loading buffer with dyes:

250mM Tris-HCl (pH 7.5)	5mL of 1M Tris-HCl, pH 7.5
0.2% bromophenol blue	0.04g bromophenol blue
40% glycerol	8mL glycerol
mQ H ₂ O to 20mL	

poly(dI-dC):poly(dI-dC) -Amersham#27-7880-01 for 10A260U i.e.~500μg

10X binding buffer (200mL):

500mM KCl (MW 74.56)	7.456g
100mM HEPES (MW 249.3)	4.99g
1mM EDTA	0.4mL of 0.5M EDTA, pH 8.0
Adjust pH to 7.9, adjust volume with mQ H ₂ O, filter-sterilize	
Add PMSF to 2.5mM final just prior to use	

10X antibody binding buffer (200mL):

1M NaCl (MW 58.44)	11.688g
100mM HEPES (MW 249.3)	4.99g
1mM EDTA	0.4mL of 0.5M EDTA, pH 8.0
30mM MgCl ₂ (MW 203.3)	1.2198g
1mg/mL BSA	2mL from 100mg/mL stock
Adjust pH to 7.9, adjust volume with mQ H ₂ O, filter-sterilize	
Add PMSF to 2.5mM final just prior to use	

Appendix N - Determination of ³⁵S incorporation in TCA precipitable fraction

This procedure is adapted from Miller and Carrino, 1980, JBC, 255(11): 5490-500. This is to monitor the incorporation of radioactivity into total cellular proteins. Proteins are precipitated with TCA using BSA as a carrier protein.

Note: Samples to be assayed are in lysis buffer, 10mM Tris-HCl, 1% Triton X-100, 1% deoxycholate, antiproteolytic agents cocktail 1 and 2

BUFFERS:

- 1mg/mL BSA (ELISA grade) (make 100mg/mL stock i.e. 5g/100mL, then 1:100 dilution i.e. 0.5mL and fill to 50mL)
 - 100mM L-methionine (MW 149.2 – 100mM solution is 1.492g/100mL)
 - 100% Trichloro-acetic acid
 - 0.1M NaOH (MW 40.0 – 0.1M is 1g/250mL)
 - 10% Trichloro-acetic acid
1. Prepare 22.5mL total (or adjust proportionally as needed) of:
 - ✓ 2.5mL (1mg/mL BSA),
 - ✓ 250μL (100mM Met),
 - ✓ 19.75mL dH₂O
 2. Aliquot 0.9mL/tube of the mix prepared in step 1 to the 5μL/tube aliquots of each cell lysate (after sonication but before centrifugation) or 5μL of blank lysis buffer
 3. Add 0.1mL/tube of 100% TCA
Note: final concentrations in tubes are 10%TCA, 0.1mg/mL BSA (used as a carrier) and 1mM methionine
 4. Chill on ice for 30-60 minutes – or overnight
 5. Eppendorf centrifugation for 10-15 min at 13,000 rpm, at 4C
 6. Remove supernatant
 7. Dissolve in 75μL 0.1M NaOH
 8. Add 1mL of 10% TCA
 9. Chill on ice for 30-60 minutes
 10. Eppendorf centrifugation for 10 minutes at 13,000 rpm, at 4C
 11. Remove supernatant, be cautious not to aspirate the pellet but try to remove as much as possible such as the remaining acid will not neutralize the base added in the next step
 12. Dissolve in 200μL 0.1M NaOH (add 50-100μL more/tube if pellets do not dissolve – do not add more than needed as the NaOH can cause some quenching)
 13. Following these 2 precipitations the solubilized protein is transferred to scintillation vials filled with 10mL scintillation fluid rinsing the tubes three times with 150μL dH₂O
 14. Count.

Appendix O - Preparation of rHL-conjugated Immunobead Matrix

Day 1

1. Make all required buffers as indicated on the manufacturer's protocol, store at 4°C
2. Dialyze 5mg antibody overnight vs. 6L coupling buffer (0.003 M phosphate, pH 6.3)

Day 2

Coupling

1. Re-hydrate the 200mg matrix bottle with 20mL distilled water. This makes a 10mg/mL suspension of coupling buffer plus 0.01% NaN₃.
2. Remove 10mL of the suspension with a sterile 10cc syringe and transfer to a 50mL conical tube.
3. Wash the matrix twice to remove the azide i.e. add coupling buffer, resuspend, centrifuge at 1,500g for 10 minutes, decant supernatant, repeat.
4. After the 2nd spin, resuspend the beads in approximately 3mL coupling buffer, add the dialyzed antibody, and adjust the volume to 10mL with coupling buffer.
5. Incubate for 1 hour at 4°C.
6. Add 20mg EDAC (BioRAD) to the mixture (stored in dessicator at 4°C). Mix very well.
7. Incubate for at least 3 hours at 4°C.

Wash (keep buffers on ice during washes)

8. Divide material into two 50mL conical tubes for wash procedure.
9. Fill and balance tubes with PBS.
10. Spin at 1,500g for 10 minutes.
11. Decant the supernatant (by inverting the tube immediately after the spin), resuspend in a small volume of PBS, vortex vigorously, fill up the tubes with PBS, and mix.
12. Spin and remove supernatant.
13. Resuspend the pellet in a small volume of 1.4 M NaCl-PBS. Fill the tubes to capacity, mix, balance and spin as before.
14. Decant and drain briefly.
15. Repeat the high salt wash.
16. Spin, and decant.
17. Repeat the PBS wash above twice.
18. Resuspend in PBS and leave at 4°C for at least 3 hours to renature the antibody.
19. Spin and resuspend the beads in 0.005 M phosphate, pH 7.2, pool the beads and spin.
20. Wash beads twice (total) with the 0.005 M phosphate, pH 7.2 and resuspend in 0.005 M phosphate-1% BSA buffer, 10mL final volume. Add 0.02% NaN₃ when solution is stored (that is add 40μL of a 5% NaN₃ stock solution (1:250 dilution)).
21. Store at 4°C for months and/or at -20°C for several years.

Appendix P - Cytoplasmic cell extract preparation

This protocol is adapted from Yukht et al., *J. Clin. Invest.*, **96**:2438-44, 1995, who cited an original protocol by Walden et al., *JBC*, **264**: 13765-9, 1989, and from Current protocols in molecular biology, pp.12.1.1-9.

BUFFERS AND REAGENTS

Lysis buffer (pH 7.4)

(50mM Tris-HCl, 35mM KCl, 10mM MgCl₂, 250mM sucrose, 0.5mM EDTA, 7mM β-mercaptoethanol)

<u>component</u>	<u>final</u>	<u>FW</u>	<u>for 500mL</u>
Tris-HCl	50mM	157.6	3.94g
KCl	35mM	74.56	1.3048g
MgCl ₂	10mM	203.3	1.0165g
Sucrose	250mM	342.3	42.788g
EDTA	0.5mM		0.5mL of 0.5M stock

Weigh powders in 50mL RNase free conicals. Fill with DEPC-treated mQH₂O, and transfer to baked graduated container, adjust pH to 7.4 with pH paper strips and adjust final volume to 500mL with DEPC mQH₂O

Autoclave

Store at 4C

Just prior to use, to the desired amount of buffer add:

β-mercaptoethanol	7mM	78.13	0.4895μL/mL
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Note: commercial stock is 14.3 moles/L

Resuspension buffer (pH 7.4)

(20mM Tris-HCl, 20mM KCl, 10% glycerol, 7mM β-mercaptoethanol)

<u>component</u>	<u>final</u>	<u>FW</u>	<u>for 1L</u>
Tris-HCl	20mM	157.6	3.152g
KCl	20mM	74.56	1.491g
Glycerol	10%		100mL

Weigh powders in 50mL RNase free conicals. Fill with DEPC-treated mQH₂O in baked glassware, adjust pH to 7.4 with pH paper strips and adjust final volume to 1L with DEPC mQH₂O

Store at 4C

Just prior to use, to the desired amount of buffer add:

β-mercaptoethanol	7mM	78.13	0.4895μL/mL
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Buffer for dialysis (pH 7.4)

(20mM Tris-HCl, 20mM KCl, 0.1mM EDTA, 10% glycerol, 7mM β -mercaptoethanol)

<u>component</u>	<u>final</u>	<u>FW</u>	<u>for 4 L</u>
Tris-HCl	20mM	157.6	12.608g
KCl	20mM	74.56	5.965g
EDTA	0.1mM		0.8mL of 0.5M stock
Glycerol	10%		400mL

Weigh powders in 50mL RNase free conicals. Fill with autoclaved mQH₂O in baked glassware, adjust pH to 7.4 and adjust final volume to 4L with mQH₂O

Store at 4C

Just prior to use, to the desired amount of buffer add:

β -mercaptoethanol	7mM	78.13	0.4895 μ L/mL
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PROCEDURE

The entire procedure should be achieved in one day, without delay. In particular, dialysis (if done) should be as short as possible.

Day before:

1. Pre-chill the SW65Ti rotor at 4C
2. Split cells 1:2 such as cells are confluent (but not overgrown) the next day

Day of:

3. Prepare control and treated cells for experiment (use 7x100mm dishes/condition):
4. Optional: Clean 3 centrifuge tubes (Seton 7022, 13x51mm, polyclear) for the SW65Ti rotor using RNAzap and then rinse with DEPC mQH₂O, invert to dry
5. Wash the cells with ice-cold PBS and aspirate the PBS off the cells
6. Add 2mL/dish fresh PBS and scrape the cells. Pool all cells from one treatment together in a 15mL graduated conical centrifuge tube

This procedure should be performed at 0-4C, in cold room. Use prechilled buffers and equipment/rotors.

7. Pellet the cells by centrifuging 10min at 3000rpm (IEC centrifuge) at 4C
8. Decant the supernatants and discard
9. Measure the packed cell volume (pvc) using graduations on tube
10. Add 3 last components to the desired volume of hypotonic lysis buffer (needed in next three steps)
11. OPTIONAL: if at all, RAPIDLY resuspend the cell pellets in a volume of lysis buffer ~5 times the pvc. Spin 5 minutes at 3000rpm (IEC centrifuge)
Note: this step removes salt from the PBS such as more swelling can occur in the next steps, it needs to be performed quickly because swelling will begin at this step already and proteins which leak out of the cells will be discarded in the supernatant
12. Discard supernatant

13. Resuspend the packed cells in hypotonic buffer to final volume of 3 times the original pvc and allow to swell 10 minutes on ice. The cells should swell at least 2 fold.
14. Homogenize in a baked glass homogenizer with 10 up and down strokes on ice, perform the homogenization slowly, particularly the down strokes
Note: an aliquot can be taken so lysis can be observed under the microscope using Trypan blue, and should be >80-90%
15. Transfer cells to RNase free 1.5mL eppendorf tubes
16. Pellet mitochondria and nuclei at 10,000g for 15 minutes at 4C
17. The supernatant is used for isolation of the S-100 fraction (high speed supernatant fraction):
 - a. Spin at 100,000g (37,000rpm) for 1-2 hours at 4C in the ultracentrifuge using the SW65Ti rotor (fits 3x5mL tubes, can go up to 300,000g if tubes are filled at least up to 1/2cm from top) and tubes (Seton 7022, 13x51mm, polyclear)
 - b. Add solid ammonium sulfate to the cytosolic S-100 fraction to 60% saturation (that is add 36.1g ammonium sulfate per 100mL solution)
 - c. Precipitate for 30min on ice
 - d. Collect precipitated proteins by centrifuging at 6,000g for 10 minutes at 0C (or 4C if 0 is not achievable) in RNase free 1.5mL eppendorf tubes
 - e. Redissolve the pellet in 300µL resuspension buffer (Ranganathan et al., *JBC*, **277**: 38669, 2002). Alternatively, redissolve the pellet and dialyze against 50 volumes of dialysis buffer (dialyze the extract for the minimum amount of time – one can check when to stop by measuring the conductivity of the extract (5-10µL/mL water) vs. that of a similar dilution of the dialysis buffer).
18. Aliquot in tubes (including aliquots for protein assay)
19. Snap freeze in liquid nitrogen or dry ice + ethanol and store at -80C
20. Determine protein concentration by Lowry (after DOC/TCA precipitation) using BSA as a standard

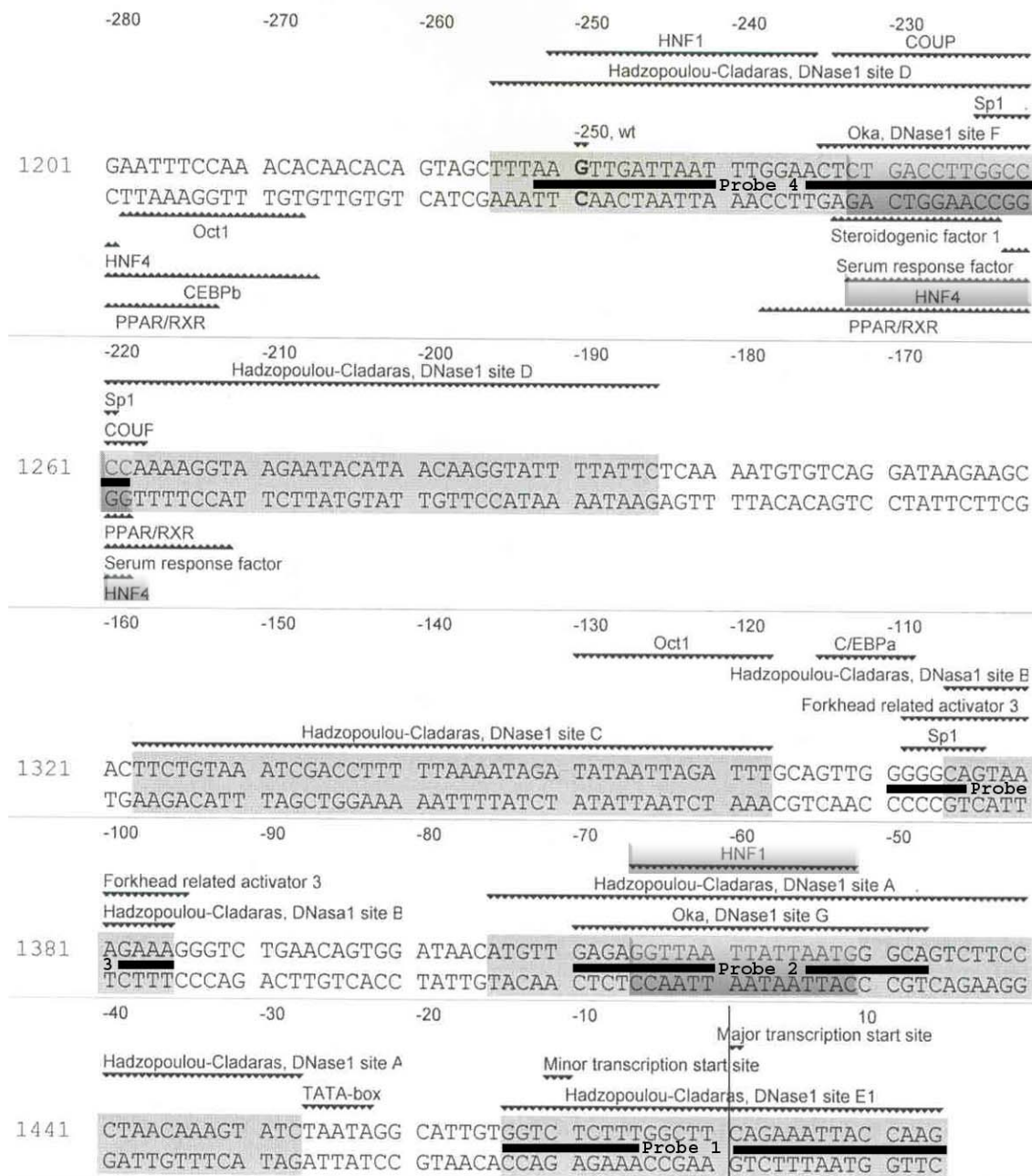
Notes:

- Observed yield was ~1mg protein/pool of 7x100mm dishes
- ~3-5 hours are needed prior to dialysis, dialysis takes ~4-6hours, do not let it go ON. After dialysis the procedure can be completed in ~1hour before freezing
- Use ~0.1µg/reaction in IVT system (rabbit reticulocyte lysate system -Promega)

Appendix Q - human HL promoter map

	-1480 Oka, DNase1 site B	-1470 Oka, DNase1 site C	-1460	-1450	-1440	-1430
1	TGTGTCAATG ACACAGTTAC	CATAAAAGTC GTATTTTCAG	AAAGTCCTTA TTTCAGGAAT	CAATGTCTCC GTTACAGAGG	CCATTCATTG GGTAAGTAAC	GCCTCCTTGC CGGAGGAACG
					NFY	
61	-1420 TATTCCTGGA ATAAGGACCT	-1410 GCACACCAGG CGTGTGGTCC	-1400 CATACTCCTA GTATGAGGAT	-1390 CCTCAGGGCC GGAGTCCCGG	-1380 TTTGACACAGA AAACGTGTCT	-1370 CTGCCCCAAG GACGGGCTTC
				HNF4		
121	-1360 CTCTCCTCCC GAGAGGAGGG	-1350 CAAGACGTCC GTTCTGCAGG	-1340 ACAGGGGCAA TGTCCCCGTT	-1330 CTCCTTCACT GAGGAAGTGA	-1320 TCCTTCAAAC AGGAAGTTTG	-1310 CTTTGCTGAA GAAACGACTT
					HNF1	
181	-1300 AGTTTAACTT TCAAATTGAA	-1290 CCCAATGCAA GGGTACGTT	-1280 TTCACCTCTAA AAGTGAGATT	-1270 CCACTCTGCT GGTGAGACGA	-1260 TAAAATCCCT ATTTTAGGGA	-1250 ACCTTCTCCC TGGAAGAGGG
		Oct1				
241	-1240 CAACCCCTG GTTGGGGGAC	-1230 CTTTTCTTGT GAAAAGAACA	-1220 AGTGGTTCCC TCACCAAGGG	-1210 ATTACATTCT TAATGTAAGA	-1200 ACCAGTCTAC TGGTCAGATG	-1190 CTAATTTTTT GATTAAAAAA
301	-1180 TTTTTTTTTT AAAAAAAAAA	-1170 TTTTGAGACG AAAACCTCTGC	-1160 GAGTCTCTCT CTCAGAGAGA	-1150 GTGTCGCCCA CACAGCGGGT	-1140 GGCTGGAGTA CCGACCTCAT	-1130 CAATGGCGCG GTTACCGCGC
361	-1120 ATCTCAGTTC TAGAGTCAAG	-1110 ACTGCAACCT TGACGTTGGA	-1100 CCGCCTCCCA GGCGGAGGGT	-1090 GGTTCAAGCG CCAAGTTCGC	-1080 ATTCTCCTGC TAAGAGGACG	-1070 CTCAGCTTCC GAGTCGAAGG
			Sp1			
421	-1060 TGAGTAGCTG ACTCATCGAC	-1050 GGATTACAGG CCTAATGTCC	-1040 CACTCACCTC GTGAGTGGAG	-1030 CACGCCCAGC GTGCGGGTCG	-1020 TAACTTTTGT ATTGAAAACA	-1010 ATTTTATAGTA TAAAAATCAT
			Ap1			
481	-1000 CAGATGGGGT GTCTACCCCA	-990 TTCACCATGT AAGTGGTACA	-980 TGGTCAGGCT ACCAGTCCGA	-970 GGTCTCGAAC CCAGAGCTTG	-960 TCCTGACCTC AGGACTGGAG	-950 GTGATCCGCC CACTAGGCGG
			Estrogen receptor			
541	-940 CTCCTCGCCT GAGGAGCGGA	-930 CCCAAAGTGC GGGTTTCACG	-920 TGTGATTACA ACACTAATGT	-910 GGAGTGAGCC CCTCACTCGG	-900 ACCGCTCCTG TGGCGAGGAC	-890 GCCAGAAATC CGGTCTTTAG
		Sp1	Forkhead related activator 4			
601	-880 TCTTCTTTAT AGAAGAAATA	-870 TATGTCTACT ATACAGATGA	-860 GTCCGTTATC CAGGCAATAG	-850 CAACTCCAGA GTTGAGGTCT	-840 AGGTAAGAAC TCCATTCTTG	-830 CTCCACTGAT GAGGTGACTA
661	-820 ACATAAGGAC TGTATTCCCTG	-810 TTGTATACCC AACATATGGG	-800 CACGTGCCTG GTGCACGGAC	-790 CAACAGTGCT GTTGTCACGA	-780 TGGCACCTAG ACCGTGGATC	-770 TAGGCATACC ATCCGTATGG
			Ebox USF			-763 _{wt}

	-760	-750	-740	-730	-720	-710 -710, wt
						HNF3b
721	AAAATATATA TTTTATATAT	AATGTTGAAC TTACAACCTG	AAATGAAGAA TTTACTTCTT	AGTTAAAGTA TCAATTTTCAT	AAACTAGAGG TTTGATCTCC	TCCAAAAATA AGGTTTTTAT
		Oct1				HNF1
	-700 HNF3b	-690	-680	-670	-660	-650
		Yin and Yang				
781	TCACAAAAGC AGTGTTCCTG	CATCTATGGT GTAGATACCA	CGCCTTTTCC GCGGAAAAGG	CTACCTGATT GATGGACTAA	TTGCTGAGTG AACGACTCAC	GCCTTACTTT CGGAATGAAA
	HNF1		PPAR/RXR			
		Serum response factor 1				
	-640	-630	-620	-610	-600	-590
			HNF1			AP1
					Oka, DNase1 site D	
841	TCAGTCCTCT AGTCAGGAGA	ACACAGCTGG TGTGTCGACC	AACATTAATG TTGTAATTAC	AACACAGAGG TTGTGTCTCC	GGAAGAAGT CCCTTCTTCA	GTGTTTACTC CACAAATGAG
					Probe 8	Forkhead related activator
	-580	-570	-560	-550	-540	-530
				Steroidogenic factor		
	Oka, DNase1 site		Oka, DNase1 site E			
	AP1	Ebox SREBP1	Ebox USF		HNF3b	
901	TAGGATCACC ATCCTAGTGG	TCTCAATGGG AGAGTTACCC	TCACCTGGCA AGTGAACCGT	AGGGCATCTT TCCCGTAGAA	TGCTTCTTCG ACGAAGAAGC	TCAGCTCCTT AGTCGAGGAA
	Forkhead related activator	AP1			AP1	CREB
	-520	-510	-500	-490	-480	-470
	-514, wt					
	Ebox USF				CEBP	NFY
961	TTGACACGGG AACTGTGCCC	GGTGAAGGGT CCTCTCCCA	TTTCTGCACC AAAGACGTGG	ACACTTTGAC TGTGAAACTG	CACAAGCATC GTGTTCGTAG	ACCAATTTCA TGGTTAAAGT
	Probe 6			HNF4		
	-460	-450	-440	-430	-420	-410
					Sp1	
1021	CTGAACCCAA GACTTGCGTT	CAGAAATTTG GTCTTTAAAC	GACCCTCTGG CTGGGAGACC	GGGCTCTCTG CCCGAGAGAC	CGTGGCAGGG GCACCGTCCC	CCCTTTTCTT GGGAAAAGAA
		HNF4			Ebox	
	-400	-390	-380	-370	-360	-350
1081	TTTCTTTGGG AAAGAAACCC	CTTAGGCTGC GAATCCGACG	AATTTGAAAC TTAAACTTTG	ACCACTTTCC TGGTGAAGAG	TGAGCCAGCA ACTCGGTCGT	TCCCCCTTGC AGGGGGAACG
	-340	-330	-320	-310	-300	-290
			SREBP half site			
			SREBP half site			
		Sp1		USF	SREBP half site	
1141	AGCGCTGTCA TCGCACAGT	CAGGGAGGCT GTCCCTCCGA	TAGGCAGCCA ATCCGTCGGT	CGTGGAAGCC GCACCTTCGG	ACCTACCCCG TGATGGGGC	ACCTTTGGCA TGGAAACCGT
			Probe 5			PPAR/RXR
						HNF4



Legend:

Putative transcription factor binding sites (Matinspector (1)), and common hHL promoter variants are indicated

Known DNase1 covered sites (2-3) are highlighted in grey



Probe used in gel-shift assays



Confirmed transcription factor binding site (by supershift assays)

(1) Quandt et al., Nucleic Acids Res. 1995;23:4878-84.

(2) Hadzopoulou-Cladaras et al., Biochemistry 1993;32:9657-67.

(3) Oka et al., Gene 1996;180:69-80.

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